Universidade de Aveiro Departamento de Biologia
Ano 2015

Lia Filipa Alvarez Pereira da Mota e Costa

Impacto dos canabinóides na gravidez: efeitos em células trofoblásticas

Cannabinoids impact on pregnancy: effects in trophoblast cells

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
Ano 2015

Lia Filipa Alvarez Pereira da Mota e Costa

Impacto de canabinóides na gravidez: efeitos em células trofoblásticas

Cannabinoids impact on pregnancy: effects in trophoblast cells

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Georgina Lopes Correia da Silva, Professora Auxiliar do Laboratório de Bioquímica, Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto, do Doutor Bruno Miguel Reis Fonseca, Investigador de Pós-doutoramento do UCIBIO/REQUIMTE, Laboratório de Bioquímica, Departamento de Ciências Biológicas da Faculdade de Farmácia da Universidade do Porto, e do Doutor Mário Jorge Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

O júri

Presidente	Maria de Lourdes Pereira Professora Associada c/Agregação, Departamento de Biologia, Universidade de Aveiro
Vogal – orientador	Georgina Lopes Correia da Silva Professora Auxiliar do UCIBIO/REQUIMTE, Laboratório de Bioquímica, Departamento de Ciências Biológicas da Faculdade de Farmácia, Universidade do Porto
Vogal – arguente principal	Elisa Oliveira Braga Keating Professora Auxiliar convidada, Departamento de Bioquímica, Faculdade de Medicina, Universidade do Porto

Agradecimentos

Porque este trabalho não seria possível sem a ajuda dos que se seguem, cabe-me agradecer a todos os que contribuíram:

À Doutora Georgina Correia-da-Silva por me ter ensinado a fazer e partilhar ciência. O seu entusiasmo contagiante foi essencial para manter a persistência, mesmo quando as coisas correram menos bem. Obrigada por me apoiar e incentivar a perseguir os meus sonhos! À Doutora Natércia Teixeira por tão bem me ter recebido e me ter dado o gosto de pertencer a esta família, pelo seu carinho e disponibilidade e por sempre ter acompanhado o meu trabalho com todo o interesse. Foi um orgulho ser uma das "suas meninas". Ao Doutor Bruno Fonseca por todo o apoio, confiança e paciência. Ao Doutor Mário Pereira, obrigada por me ter acompanhado desde o início e por sempre ter incentivado e valorizado o meu trabalho. A todos vós, muito obrigada pela vossa orientação, por terem confiado em mim e por me terem feito crescer como cientista e pessoa. Foi uma honra ser vossa aluna.

Ao Doutor Jorge Braga e Doutora Alexandrina Mendes, do Centro Materno-Infantil do Norte – Centro Hospitalar do Porto, pela preciosa parceria.

À Doutora Mariana Costa, muito obrigada por me teres "batizado" no mundo da Ciência. Todos os teus ensinamentos foram muito enriquecedores.

À Doutora Cristina Amaral, "mais que nossa mãe", pela incansável ajuda. Devo-te um jantar! À (quase) doutora Marta Almada, pelas palavras certas nos momentos certos. Ambas foram o meu suporte ao longo deste ano. Obrigada pelos ensinamentos, chamadas de atenção, palavras de apoio, por ouvirem os meus desabafos, pelas gargalhadas e amizade!

À Doutora Susana Rocha e Sandra Ribeiro que amavelmente me socorreram sempre que precisei.

Aos restantes colegas, Filipa Sobral, Carina Brites, Rafael Fernandes, Renata Fernandes, João Maia, Tiago Augusto e Fernanda Bernardo, obrigada pelo companheirismo e muita sorte para todos!

À Ana Paula e à Dona Casimira, restantes professores e alunos do laboratório de Bioquímica da Faculdade de Farmácia da Universidade do Porto, porque todos contribuíram para que todos os dias fosse trabalhar com um sorriso no rosto. Prometo voltar com frutos e docinhos!

Às minhas queridas colegas de casa e companheiras de aventuras Beatriz Pinto, Beatriz Natário e Anabela Azevedo, por me terem animado nos dias menos bons e por terem partilhado a euforia de cada dia bem sucedido.

Aos meus pais, a quem dedico este trabalho, porque sem eles não seria quem sou. Deixar-vos orgulhosos é a maior compensação! Sei que o cansaço e por vezes o desânimo vos preocupou mas, no fim, todas as conquistas fazem valer a pena. Obrigada por todo o vosso apoio e amor. Por fim, resta-me agradecer à restante família e amigos, pelo apoio, principalmente aos meus avós, por serem os mais “babados”!

Palavras-chave: Fitocanabinóides; Canabinóides Sintéticos; Endocanabinóides; Sistema Endocanabinóide; Placenta; Citotrofoblastos

Resumo

Os canabinóides (CBs) podem ser classificados como: fitocanabinóides, os constituintes da planta *Cannabis sativa* L.; canabinóides sintéticos, sintetizados em laboratório e os endocanabinóides, que são mediadores lipídicos endógenos. Os compostos canabinóides ativam recetores canabinóides – CB1 e CB2. O composto psicoativo mais prevalente é o Δ9-tetrahidrocanabinol (THC), mas mais de 60 diferentes CBs foram já identificados a partir da planta. Os endocanabinóides (eCBs) melhor caracterizados são a anandamida (AEA) e o 2-araquidonoilglicerol (2-AG), que estão envolvidos em vários processos biológicos, incluindo plasticidade sináptica, modulação da dor, homeostasia energética e reprodução. Por outro lado, alguns canabinóides sintéticos, inicialmente projetados para investigação médica, são agora usados como drogas de abuso.

Durante o período de desenvolvimento placentário ocorrem processos de remodelação que envolvem proliferação, apoptose, diferenciação e invasão dos trofoblastos. Sabe-se que um controlo rigoroso dos níveis de eCBs é necessário para uma progressão normal da gravidez e que os eCBs estão envolvidos no *turnover* celular dos trofoblastos. Assim sendo, ao partilharem a ativação dos mesmos recetores, a exposição a exocanabinóides, seja pelo uso recreativo ou medicinal, pode levar a alterações nos níveis de eCBs e na homeostasia do sistema endocanabinóide (ECS).

Neste trabalho foi estudado o impacto dos CBs em células trofoblásticas BeWo e em culturas primárias de citotrofoblastos humanos. As células foram tratadas durante 24 horas com diferentes concentrações de THC, do canabinóide sintético WIN-55,212 (WIN) e de 2-AG. O tratamento com THC não afetou a viabilidade das células BeWo, enquanto que o WIN e o 2-AG causaram uma perda de viabilidade dependente da dose. Estudos morfológicos, juntamente com marcadores bioquímicos, indicam que o 2-AG é capaz de induzir apoptose em citotrofoblastos.

Por outro lado, estudos morfológicos realizados com laranja de acridina sugerem que a autofagia pode estar envolvida na perda de viabilidade induzida pelo WIN. Todos os canabinóides induziram perda de potencial de membrana mitocondrial (Δψm), mas apenas o 2-AG levou a um aumento na formação de ROS/RNS, sem terem sido observadas

diferenças nos níveis de glutationa. O *stress* reticular pode estar envolvido no *stress* oxidativo induzido pelo 2-AG, visto que resultados preliminares apontam para um aumento na expressão de *CCAAT-enhancer-binding protein homologous protein* (CHOP). Para além da diminuição da viabilidade celular, os resultados sugerem alterações na progressão do ciclo celular. O tratamento com WIN induziu retenção do ciclo celular em fase G_0/G_1, enquanto que o 2-AG levou a uma retenção em fase G_2/M.

Neste trabalho é reforçada a importância da sinalização canabinóide em processos importantes de proliferação e morte celular de células trofoblásticas. Visto que as drogas canabinóides são as mais consumidas a nível mundial, e umas das drogas recreativas mais consumidas pelas mulheres grávidas, este estudo pode contribuir para a compreensão do impacto destas substâncias na reprodução humana.

Keywords: Phytocannabinoids; Synthetic cannabinoids; Endocannabinoids; Endocannabinoid System; Placenta; Cytotrophoblasts

Abstract

Cannabinoids (CBs) can be classified as: phytocannabinoids, the constituents of the Cannabis sativa plant; synthetic cannabinoids lab-synthesized and the endocannabinoids that are endogenous lipid mediators. Cannabinoid compounds activate cannabinoid receptors – CB1 and CB2. The most prevalent psychoactive phytocannabinoid is Δ9-tetrahydrocannabinol (THC), but more than 60 different CBs were already identified in the plant. The best characterized endocannabinoids (eCBs) are anandamide (AEA) and 2-arachidonoylglycerol (2-AG), that are involved in several physiological processes including synaptic plasticity, pain modulation, energy homeostasis and reproduction. On the other hand, some synthetic cannabinoids that were initially designed for medical research, are now used as drugs of abuse.

During the period of placental development, highly dynamic processes of remodeling occur, involving proliferation, apoptosis, differentiation and invasion of trophoblasts. It is known that a tight control of eCBs levels is required for normal pregnancy progression and that eCBs are involved in trophoblast cells turnover. Therefore, by sharing activation of the same receptors, exposure to exocannabinoids either by recreational or medicinal use may lead to alterations in the eCBs levels and in the endocannabinoid system homeostasis

In this work, it was studied the impact of CBs in BeWo trophoblastic cells and in primary cultures of human cytotrophoblasts. Cells were treated for 24 hours with different concentrations of THC, the synthetic cannabinoid WIN-55,212 (WIN) and 2-AG. Treatment with THC did not affect BeWo cells viability while WIN and 2-AG caused a dose-dependent viability loss. Morphological studies together with biochemical markers indicate that 2-AG is able to induce apoptosis in cytotrophoblasts. On the other hand, morphological studies after acridine orange staining suggest that autophagy may take part in WIN-induced loss of cell viability. All cannabinoids caused a decrease in mitochondrial membrane potential (Δψm) but only 2-AG led to ROS/RNS generation, though no changes in glutathione levels were observed. In addition, ER-stress may be involved in the 2-AG induced-oxidative stress, as preliminary results point to an increase in *CCAAT-enhancer-binding protein homologous protein* (CHOP) expression. Besides the decrease in cell viability, alterations in cell cycle

progression were observed. WIN treatment induced a cell cycle arrest in G0/G1 phase, whereas 2-AG induced a cell cycle arrest in G2/M phase.

Here it is reinforced the relevance of cannabinoid signaling in fundamental processes of cell proliferation and cell death in trophoblast cells. Since cannabis-based drugs are the most consumed illicit drugs worldwide and some of the most consumed recreational drugs by pregnant women, this study may contribute to the understanding of the impact of such substances in human reproduction.

Table of contents

Chapter II

Material and Methods **Page**

Chapter III

Results **Page**

Index of figures

Index of tables

List of abbreviations

2-AG – 2-arachidonoylglycerol

AB-AM – antibiotic-antimycotic solution

AEA – anandamide, N-arachidonoylethanolamine

AO – acridine orange

AVOs – acid vesicular organelles

CB – cannabinoid

CB1/CB2 – cannabinoid receptor 1/2

CBD – cannabidiol

CBN – cannabinol

CCCP – carbonyl cyanide m-chlorophenylhydrazone

c-FLIP – FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein

CHOP – CCAAT-enhancer-binding protein homologous protein

CNS – central nervous system

COX-2 – cyclooxygenase-2

CP 47,497 – 2-[(1R,3S)-3-hydroxycyclohexyl]-5-(1,1-dimethylnonyl)phenol

CT – cytotrophoblasts

DAG – 1,2-diacylglycerol

DAGL – diacylglycerol lipase

DCDHF-DA – 2,7-dichlorodihydrofluorescein diacetate

$DiOC_6$ – 3,3′-dihexyloxacarbocyanine iodide

DMEM – dulbecco's modified eagle medium

DMSO – dimethyl sulfoxide

DTNB – 5,5′-Dithiobis(2-nitrobenzoic acid)

eCB – endocannabinoid

ECS – endocannabinoid system

EDTA – ethylenediaminetetraacetic acid

egEVTs – endoglandular extravillous trophoblasts

EMT – endocannabinoid membrane transporter

enEVTs – endovascular extravillous trophoblasts

ER – endoplasmic reticulum

ERK – extracellular regulated kinase

EVTs – extravillous trophoblasts

FAAD – Fas-associated protein with death domain

FAAH - fatty acid amide hydrolase

FAK – focal adhesion kinase

FAN – adaptor protein factor associated with neutral sphingomyelinase activation

Fas – Fas cell surface death receptor

FBS – fetal bovine serum

GPCR – G protein-coupled receptors

GPR55 – G-protein-coupled receptor 55

GPx – glutathione peroxidase

GSH – reduced glutathione, sulfhydryl form glutathione

GSSG – oxidized glutathione, glutathione disulfide

HU-210 – 3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol

IAPs – inhibitors of apoptosis proteins

iEVTs – interstitial extravillous trophoblasts

IgG – immunoglobulin G

JNK – c-Jun N-Terminal Kinases

JUN – jun proto-oncogene

JWH-018 – 1 Naphthalen-1-yl-(1-pentylindol-3-yl)methanone

LDH – lactate dehydrogenase

MAG – monoacylglycerols

MAGL – monoacyl glycerol lipase

PARP – poly (ADP-ribose) polymerase

PKA – protein kinase A

ST – syncytiotrophoblasts

STS – staurosporine

THC – Δ9-tetrahydrocannabinol

TNB – 5-thio-2-nitrobenzoic acid

TNF – tumor necrosis factor

TRIB-3 – tribbles pseudokinase 3

TRPV1 – transient receptor potential vanilloid 1

WIN – WIN-55,212, (R)-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo

[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt

Z-VAD-FMK – fluoromethylketone

Δψm - mitochondrial membrane potential

Author's publications/ communications

Lia Costa, Cristina Amaral, Natércia Teixeira, Georgina Correia-da-Silva, Bruno Miguel Fonseca, *Cannabinoid-induced autophagy: protective or death role? **

* Manuscript submitted to *Prostaglandins and Other Lipid Mediators*. Under second revision.

(The point *2.3 Cannabinoids and autophagy* in the Introduction chapter is part of this manuscript, which full version is included in appendix)

Costa L.F., Costa M.A., Fonseca B.M., Mendes A., Pereira M., Teixeira N.A., Correia-da-Silva G., *Endocannabinoids impact in trophoblast turnover: 2-arachidonoylglycerol induces reactive oxygen/nitrogen species generation and cell death in primary cultures of cytotrophoblasts*, *Human Reproduction*, vol 30, supp 1, 2015, pp 188-189 *

* (P-155) Poster presentation at the ESHRE annual meeting – European Society of Human Reproduction and Embryology, 14-17June, 2015, Lisbon, Portugal

Costa L.F., Costa M.A., Fonseca B.M., Pereira M., Teixeira N.A., Correia-da-Silva G., *Efeito do endocanabinóide 2-araquidonilglicerol na placenta humana: alterações do estado oxidativo dos citotrofoblastos *.* (C.O.49) Oral communication at the IV Encontro Nacional de Pós-Graduação em Ciências Biológicas, 30 March-2 April, 2015, Aveiro, Portugal.

* Selected for extend abstract publication in the online national journal CAPTAR

L.F. Costa, B.M. Fonseca, C. Amaral, A. Mendes, J. Braga, N.A. Teixeira, G. Correia-da-Silva, *Cannabinoid signaling in human placenta: effects of cannabinoids in cytotrophoblast cells *.* (CO0047) Oral communication at the UMIB summit 2015 – Unidade Multidisciplinar de Investigação em Biomedicina, 24-25 September, Porto, Portugal.

* Abstract selected for publication in a special supplement edition of the *Medicine® Journal*

Lia Costa, Bruno Fonseca, Natércia Teixeira, Georgina Correia-da-Silva, *Effects of synthetic cannabinoids in placenta: WIN-55,212 induces a mitochondrial-dependent cell death mechanism in trophoblast cells*. (PS82) Abstract selected for oral communication at the plenary session on 10th YES Meeting – Young European Scientist Meeting – 20 September, 2015, Porto, Portugal *

***** Communication awarded with the Plenary Session Honorable Mention

L.F. Costa, M.A. Costa, B.M. Fonseca, N.A. Teixeira, G. Correia-da-Silva, *Cannabinoids impact in placental cytotrophoblasts: changes in the oxidative state of BeWo cells*. (6106) Oral presentation at the IJUP'15 – Investigadores Jovens da Universidade do Porto, 13-15 May, 2015, Porto, Portugal

Chapter I

Introduction

1. Cannabinoids and the endocannabinoid system

Cannabinoid compounds can be divided in three types: (a) phytocannabinoids, found in the *Cannabis sativa* L. plant, such as Δ9-tetrahydrocannabinol (THC), (b) the laboratory synthesized cannabinoids, the synthetic compounds and (c) endogenous lipid compounds called endocannabinoids [1]. Cannabinoids are able to activate cannabinoid receptors, CB1 and CB2 [2], although they can be ligands of other receptors.

CB1 receptors are more abundant in the SNC and mediate the actions of THC, like impairments in cognition, memory, learning and motor coordination [3]. CB1 is also expressed in other several organs like spleen, tonsils, bladder, small intestine, sympathetic nerve terminals, smooth muscle cells and reproductive tissues. CB2 receptors, which share only 44% of amino acid sequence homology with CB1 and are evolutionarily divergent [4], are primarily found on cells of the immune system, but also found in CNS and reproductive organs [5].

1.1 Phytocannabinoids

Cannabis sativa has been cultivated throughout human history and the oldest known written record on cannabis use dates to 4000 B.C, in ancient China, though it is believed that it was cultivated in Mongolia and southern Siberia as far as 12.000 years ago, which places the plant among humanity's oldest cultivated crops [6]. The plant has been used for a large variety of purposes, as a source of industrial fiber, seed oil, food, paper, clothes, recreation and medicine. Vikings and medieval Germans used cannabis for relieving pain during childbirth and for toothaches, while India’s traditional medicine has used cannabis as an hallucinogenic, hypnotic, sedative, analgesic, and anti-inflammatory agent. Medical indications for cannabis included rheumatic pain, intestinal constipation, disorders of the female reproductive system, malaria and others [7].

Over the last decades there has been a marked increase in cannabis use in developed countries including Europe and USA [8] **(figure1).** The increase in cannabis use has raised questions about the extent to which cannabis may have harmful physical or psychological effects on users.

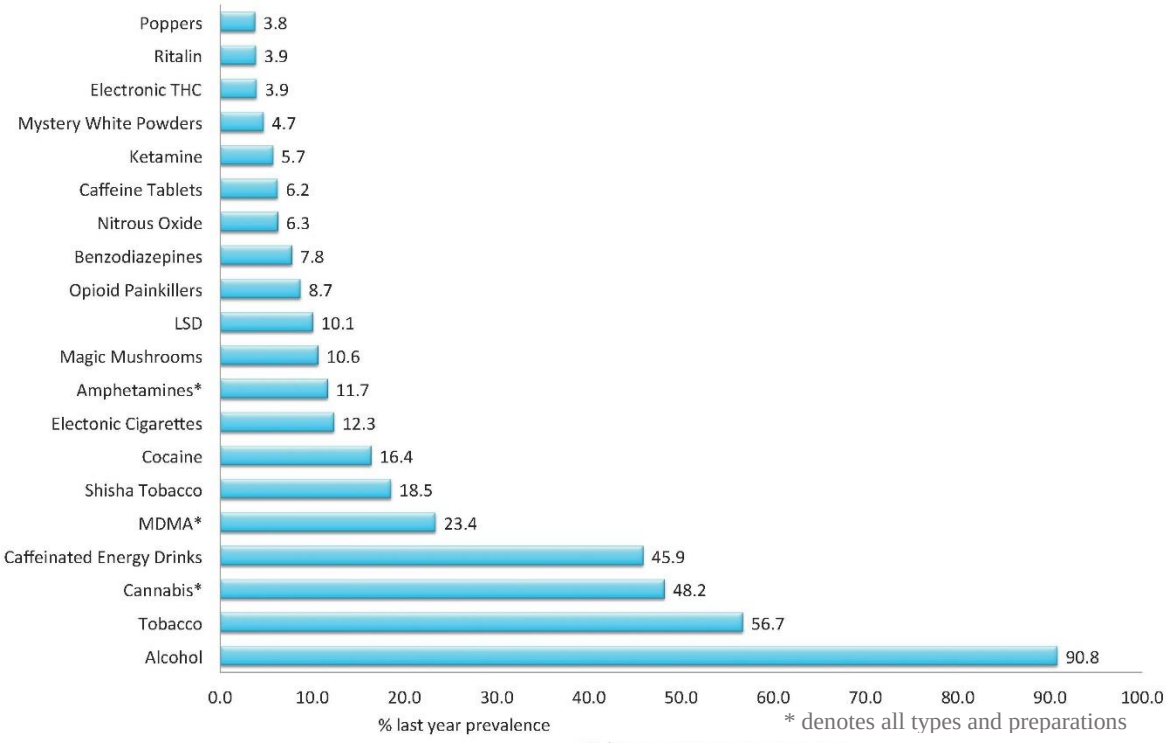

Figure 1 - Most consumed drugs/alcohol in 2014, from Global Drug Survey, [9]

Marijuana and hashish are derived from cannabis plant. The active components, known as “cannabinoids”, are found mainly within the flowers, but also in leaves, stalks, and seeds. More than 400 compounds have been identified, including 60 different cannabinoids.

The first cannabinoids identified, by Mechoulam, Shvo and Gaoni, in 1963 and 1965, were THC [10] and Cannabidiol (CBD) [11]. Another phytocannabinoid present in cannabis is Cannabinol (CBN), which has no psychoactive effects **(figure 2)**, while THC, the major active compound in marijuana, is primarily responsible for its psychoactive effects.

Nevertheless, the legalization of marijuana for medical use is still a controversial issue. Additionally, marijuana is much more powerful today than it was 30 years ago, since the average THC levels in plants rose from less than 1% in the mid-1970's to more than 6% in 2002 and 12% in 2012 [12]. Due to its sedative, analgesic and anti-inflammatory properties [13], indications for medical cannabis use include treatment of nausea and vomiting as side effects of cancer treatment [14], poor appetite in AIDS-related wasting [15], chronic pain [16] and painful spasms in multiple sclerosis [17], epileptic convulsions [18], and glaucoma [19]. To date, for the treatment of chemotherapy-induced nausea and emesis, two cannabinoid-based medications have gained approval by the Food and Drug Administration - Marinol® (dronabinol), a laboratory synthesized tetrahydrocannabinol [20]

and Cesamet® (nabilone), a synthetic cannabinoid analogue of THC [21]. Marinol can be prescribed as an appetite stimulant to treat cachexia in cancer and AIDS patients [20]. Sativex®, a drug that contains equivalent concentrations of THC and CBD, has been approved in many countries to relieve spasticity in multiple sclerosis patients and as an adjunctive analgesic treatment for adult patients with advanced cancer [22, 23].

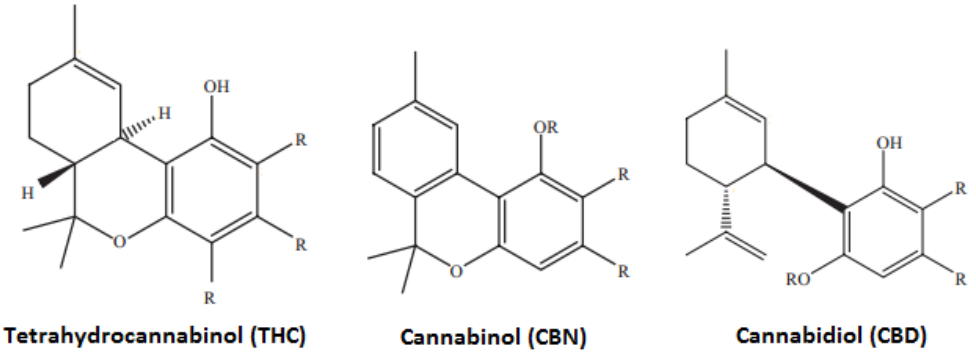

Figure 2 - Chemical structure of some phytocannabinoids. Structure of the main phytocannabinoid Tetrahydrocannabinol (THC) and other cannabinoid compounds found in the cannabis plant, Cannabinol (CNB) and Cannabidiol (CBD).

Many evidences suggest that cannabis use may negatively impact several aspect of people's lives, including mental and physical health, cognitive function, pre- and postnatal development among offspring **(table 1).** Indeed, marijuana is associated with adverse psychiatric, cardiovascular, respiratory, and immunologic events. Moreover, marijuana may interact with a number of prescription drugs and concomitant disease states [24].

Marijuana may exacerbate psychiatric disorders in patients with schizophrenia, psychosis, bipolar disorder, depression, eating disorders, or panic and anxiety disorders [25]. In addition, marijuana abusers are four times more likely to develop depression than people who do not use marijuana [26]. Long-term consumption has been associated with respiratory diseases, it may worsen chronic obstructive pulmonary disease, asthma, and tuberculosis [27]. In pregnancy, it may impair intrauterine growth and cause structural and neurobehavioral alterations in the fetus [28].

Table 1 - Possible health effects of cannabis consumption

Possible Health Effects	
Short-term	Enhanced sensory perception and euphoria followed by drowsiness/relaxation; slowed reaction time; problems with balance and coordination; increased heart rate and appetite; problems with learning and memory; hallucinations; anxiety; panic attacks; psychosis.
Long-term	Mental health problems, chronic cough, frequent respiratory infections.
Other Health-related Issues	Youth: possible loss of IQ points when repeated use begins in adolescence. Pregnancy: babies born with problems with attention, memory, and problem solving.
In combination with Alcohol	Increased heart rate, blood pressure; further slowing of mental processing and reaction time.
Withdrawal Symptoms	Irritability, trouble sleeping, decreased appetite, anxiety.

1.2 Synthetic Cannabinoids

Synthetic cannabinoids (sCBs) are often chemically related to THC and sometimes called “synthetic marijuana” or “legal marijuana”, but actually the effects can be considerably more powerful, unpredictable and more dangerous [29]. Most of these products have particularly long half-lives, which is one of the reasons why their pharmacological effects may differ significantly [30]. In fact, sCBs are anything but harmless and were never designed for human consumption, but synthesised to investigate potential medical uses of cannabis. They are full agonists of the CB receptors, and many of them have higher efficacy than THC [31, 32]. There is no published safety data for the majority of compounds, and little is known about their effects in humans. Many are controlled substances, but repeatedly modifications of the compounds produce new versions not covered by legislation [33]. The dangerous drugs are labelled as “Spice”, “K2”, “Blonde”, “Summit”, “Blaze”, “Red Dawn X”, “Citron”, “Green Giant”, “Red Giant”, “Keisha Kole”, “Geeked Up” or “Ninja”, for example, and marked as incense, herbal mixtures or potpourri in order to mask its true purpose [29]. Unfortunately, recent data from Global Drug Survey showed that the consumption of these drugs of abuse and the percentage of users in need for emergency medical treatment is dangerously rising [9, 34] **(figure 3).** The use of sCBs is linked with nausea and vomiting,

high blood pressure, tremor, hallucinations, paranoia, anxiety, psychosis, seizures, acute renal failure and, in some cases, death [29].

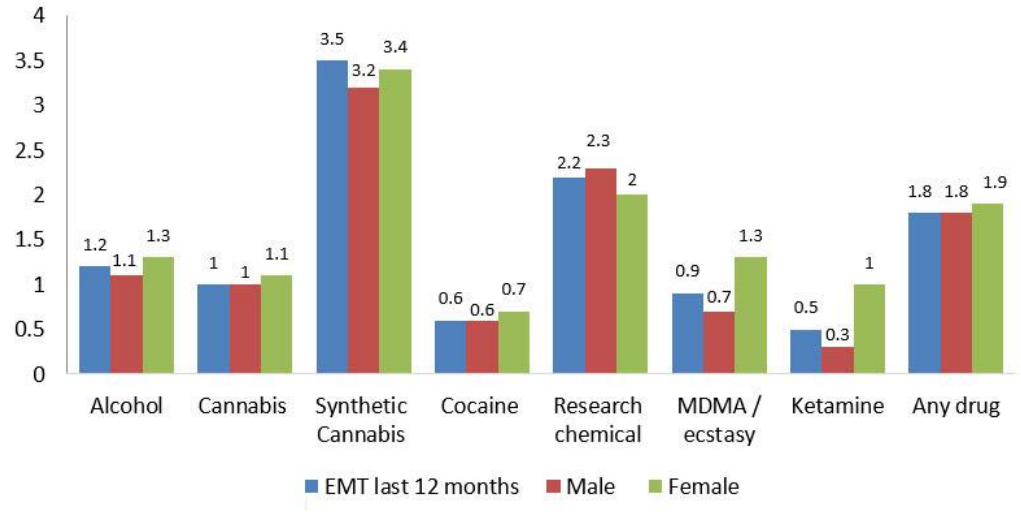

Figure 3 - Percentage of people that had sought emergency medical treatment (EMT) following the use of drugs/alcohol in 2015, from Global Drugs Survey [34]

The synthetic cannabinoids fall into three major structural groups: (i) Aminoalkylindoles (e.g. JWH-018 and WIN-55,212), the dominant cannabinoid series detected in Spice [35]; (ii) Non-classical Cyclohexylphenols (e.g. CP 47,497 and homologues); and (iii) Classical cannabinoids (e.g. HU-210) **(Figure 4)** [36].

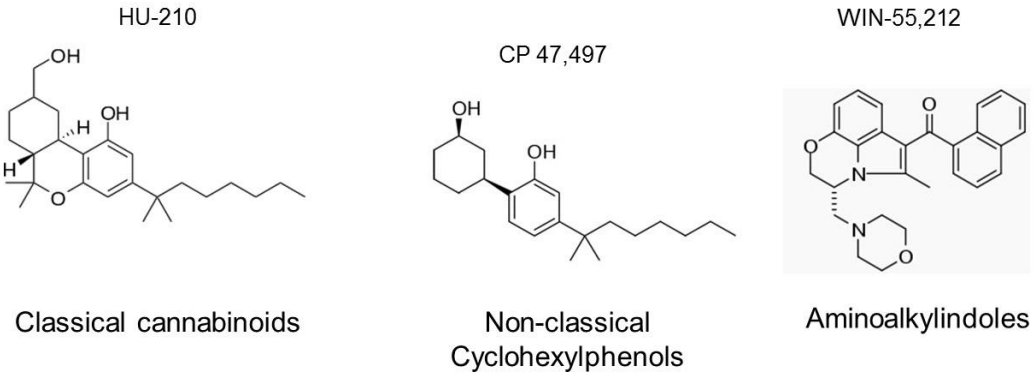

Figure 4 - Chemical structure of some synthetic cannabinoids. Representative examples.of the three classes of synthetic cannabinoids: Classical, Non-classical and Aminoalkylindoles.

A recent study about the relation between chemical structure and activity of the numerous synthetic cannabinoids concluded that the best predictor of potential cannabimimetic effects was the affinity for CB1 receptor [37]. WIN 55,212-2 is one of the most widely used analogues of cannabinoids designed to activate both CB1 and CB2 receptors [38]. It has been demonstrated that CB receptor agonists like WIN produce protective effects in the brain, including the WIN-induced reduction of epileptic manifestations in rats [39] and the WIN-induced anticonvulsant activity in pentylenetetrazole (PTZ)-evoked seizures in rats [40].

Supporting a modulatory role of synthetic cannabinoids in the toxic events elicited by toxic metabolites involved in neurodegeneration, Colín-González et al. reported that, as pre-treatment, WIN exerted protective effects on the mitochondrial dysfunction, and prevented the reactive oxygen species (ROS) formation and lipid peroxidation. These effects were induced by endogenous metabolites that accumulate in the brain of children affected by severe organic acidemias with neurodegeneration [41]. WIN also increases nociception threshold in cholestatic rats. This has implications for the treatment of the pruritus of cholestasis since pruritus is a nociceptive stimulus and drugs that increase the threshold to nociception may be a novel approach to the treatment of this symptom in patients with liver disease [42]. Another study suggests that peripherally-administered WIN may be effective in relieving cancer pain since it attenuated tumor-evoked mechanical hyperalgesia by activation of both peripheral cannabinoid 1 and cannabinoid 2 receptors [43]. Due to its anti-inflammatory properties, WIN was also able to reduce the expression of some metalloproteinases associated with cartilage degradation in human chondrocytes, suggesting a mechanism by which cannabinoids may act to prevent cartilage breakdown in arthritis [44].

1.3 Endocannabinoids

In 1992, almost 30 years after the identification of THC, Devane and collaborators identified the first endogenous compound that activates the cannabinoid receptor CB1. This compound was N-arachidonoylethanolamide and named as Anandamide (AEA), derived from the Indo-european language Sanskrit word “ananda”, which means “internal bliss” [45]. Then, in 1993, the second cannabinoid receptor CB2 [46] was found and other endocannabinoids have also been identified in the last decades, such as 2-

arachidonoylglycerol (2-AG, [47]), 2-arachidonyl glyceryl ether (noladin ether, [48]), O-arachidonoyl ethanolamine (virodhamine, [49]) and N-Arachidonoyl dopamine (NADA, [50]). AEA and 2-AG are the best characterised members of the main families of eCBs [51], N-acylethanolamides (NAE) and monoacylglycerols (MAG), respectively. The "major" endocannabinoids are represented in **figure 5.** Other substances called endocannabinoid-like compounds, are endogenously synthetized. Though they are not capable of activating the CB receptors [52], they may interfere with eCBs metabolism. Some examples are palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are present in small amounts in almost all mammalian tissues [53].

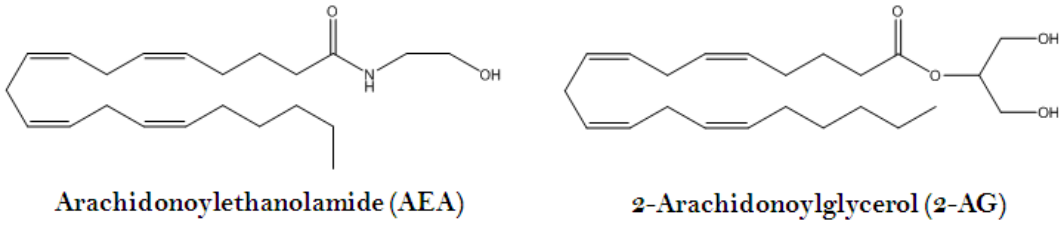

Figure 5 - Chemical structure of the major endocannabinoids 2-AG and AEA, adapted from [54]

The eCBs act as autocrine and paracrine mediators and modulate several cellular processes like proliferation, differentiation and apoptosis [55]. The eCBs have different affinities for cannabinoid receptors, different pharmacological profile comparing with THC and synthetic cannabinoids and can also bind and activate other receptors [56], such as transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator activated receptors family (PPAR). The existence of other CB receptors like G-protein-coupled receptor 55 (GPR55), the purported CB3, has been also proposed [56]. The activation of the non-selective cation channel TRPV1 by AEA can be the responsible for non-CBs mediated cellular effects such as vasodilatation and apoptosis [57]. Contrary to AEA, 2-AG seems to be a very week activator of TRPV1. At higher concentrations, AEA can also activate the nuclear receptors PPAR-α and PPAR-ϒ [58-60], and 2-AG is capable to activate PPAR-ϒ [59].

Regarding the "major" eCBs actions, it is already known that AEA is very important in processes like modulation in different stages of reproduction, analgesia, muscle relaxation, immunosuppression, inflammation, stimulation of appetite, emesis, proliferation, differentiation and apoptosis [55, 61], acting also as an endocrine messenger [60]. 2-AG seems to be an important messenger in multiple physiological processes of different

systems like nervous, immune or cardiovascular, but its exact functions still remain unclear [62].

When activated by the eCBs, the cannabinoid receptors interfere with several signalling pathways in order to exert the effects on the several organs and tissues. In the neurons, pre-synaptic stimulation of CB1 inhibits the release of neurotransmitters [63]. In the liver, stimulation of CB1 leads to an increase in acetyl-coenzyme A carboxylase and fatty acids with consequent increase in lipogenesis [64]. On the other hand, activation of CB2 seems to mediate immunosuppression effects [65]. Besides, eCB signalling is involved with several other physiological and pathophysiological processes, namely memory, pain, inflammation, apetite, reproduction, cardiovascular system, and others **(figure 6)** [66-69].

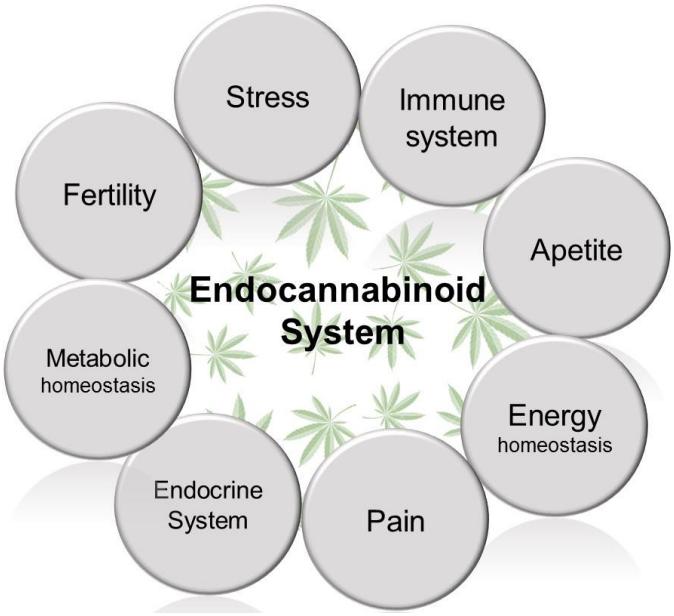

Figure 6 - Physiological and pathophysiologic processes in which eCB are involved. Adapted from [70].

Therapeutic approaches that modulate the endocannabinoid system (ECS) through the interference with eCBs levels, instead of the use of exocannabinoids, may be an advantage since it preserves the endocannabinoid specificity and limits the secondary effects [70].

1.4 Endocannabinoid System

Following the identification and synthesis of THC by Mechoulam and Gaoni in Israel (1964) [10], scientists learned a lot about the pharmacology, biochemistry and clinical effects of cannabis, but no one really knew how it worked. In 1973, when American researchers at Johns Hopkins University identified receptor sites in the brain capable of binding with opioids, such as morphine and heroin [71], some scientists expected that the discovery of receptor sites for marijuana would soon follow. In fact, 15 years later, a study at the St. Louis University School of Medicine revealed that the brain has receptor sites that respond pharmacologically to marijuana compounds. A potent THC analogue was synthesized, allowing scientists to investigate the precise locations of cannabinoid receptors in the brain [72]. The DNA sequence that encodes a THC-sensitive receptor in the rat's brain and a successful clone of marijuana receptor was announced by Lisa Matsuda in the National Academy of Science's Institute of Medicine meeting in 1990 [73, 74]. The cloning of that receptor opened the door for scientists to sculpt molecules that "fit" this receptor and develop genetically engineered "knockout" mice that lack this receptor. Then, as referred previously, the discovery of the first endogenous cannabinoid compounds and the cannabinoid receptors pointed to the existence of an endocannabinoid system (ECS). The eCBs, CB receptors, the proteins that transport, synthesizes and degrade them, compose the endocannabinoid system (ECS), represented in **figure 7**.

eCBs are released from membrane phospholipid precursors through specific phospholipases, which are activated "on demand". Although there are several biosynthetic pathways involved in the formation of AEA, the most prominent route engages a transacylation by a Ca^{2+} dependent N-acyltransferase (NAT) to produce N-arachidonoylphosphatidylethanolamine (NArPE), with the subsequent conversion of this lipid into AEA through the activity of a specific phospholipase D (NAPE-PLD). Similar to AEA, 2-AG is synthesized in a two-step pathway, which includes a rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC) to generate 1,2-diacylglycerol (DAG), sequentially converted into 2-AG by diacylglycerol lipase (DAGL). Then, the eCBs are transported out of the cell by a specific transporter, called endocannabinoid membrane transporter (EMT) and, after re-uptake through the same transporter, eCB signalling is finished by hydrolysis via fatty acid amide hydrolase (FAAH) or monoacyl glycerol lipase (MAGL). For this, hydrolases break the amide bond of AEA to release arachidonic acid and ethanolamine. On the other hand, FAAH and more importantly a specific monoacylglycerol

lipase (MAGL) transform 2-AG into arachidonic acid and glycerol. Also cyclooxygenase-2 (COX-2) is able to accept AEA as a substrate, to generate prostaglandin-ethanolamides, and can deoxygenate 2-AG to produce prostaglandin-glyceryl esters [54]**.**

The studies on the ECS are expanding to the identification of novel endogenous ligands, unveil their metabolism, the signalling pathways and their final action, as well as their potential for therapeutics.

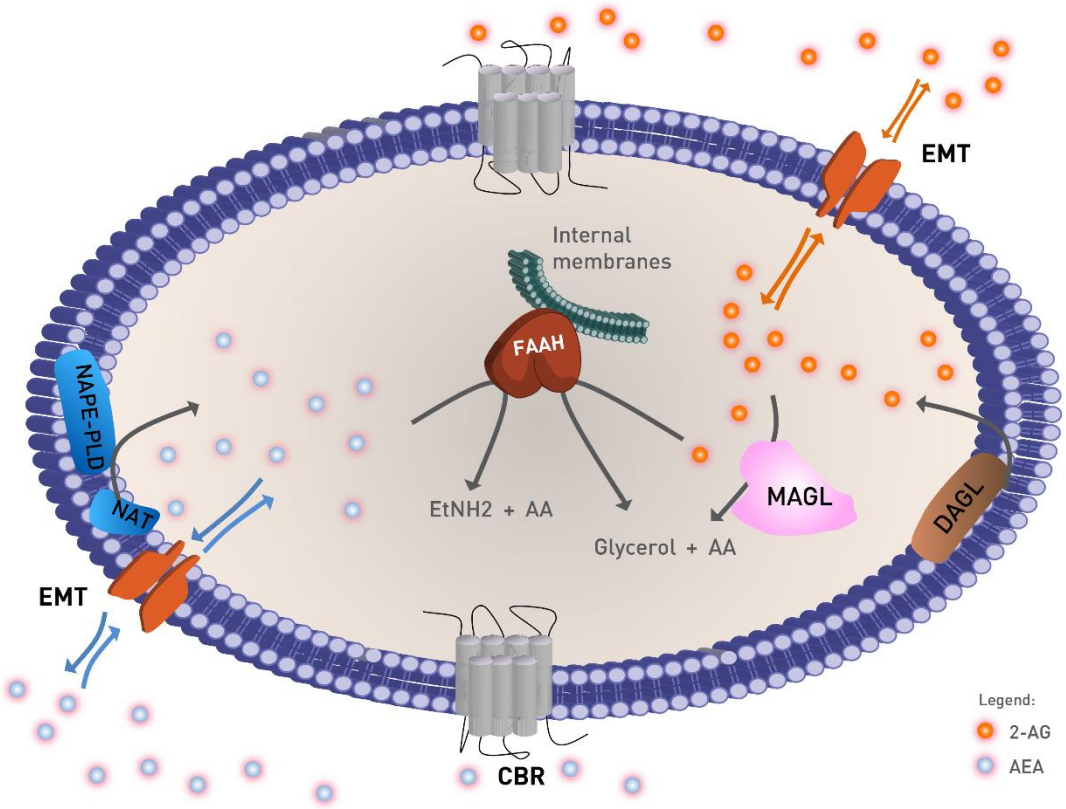

Figure 7 - Endocannabinoid system. The biosynthesis of anandamide (AEA; blue circles), from membrane precursors is catalysed by the N-acyltransferase (NAT) followed by N-acyl-phosphatidylethanolamines-specific phospholipase D (NAPE-PLD). 2-arachidonoylglycerol (2-AG; orange circles) synthesis occurs also from membrane precursors but through diacylglycerol lipase (DAGL). Endocannabinoids are transported in both directions through cell membrane by diffusion or selective transport via the putative endocannabinoid membrane transporter (EMT). In the extracellular space can interact with cannabinoid receptors (CBR) or be internalized and degraded. AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) into ethanolamine (EtNH2) and arachidonic acid (AA) or by an alternative oxidative mechanism, by cyclooxygenase-2 (COX-2) into prostaglandin-ethanolamines. 2-AG is hydrolyzed through monoacylglycerol lipase (MAGL) or FAAH into glycerol and arachidonic acid, from [54].

2. Cannabinoids and cell fate

2.1 Cannabinoid signalling pathways

Cannabinoid receptors are typical members of the G protein-coupled receptors (GPCR) family, and include CB1 and CB2 as the best characterized targets [75]. Their ligands bind through the extracellular domain, activating different cellular pathways **(figure 8).** The two CB receptors have differences in the affinity of some agonists and antagonists and in the activated signalling pathways. The physical association with lipid rafts represents another difference between CB receptors. Unlikely CB1, the binding and activation of CB2 is not dependent on the microarchitecture of the plasma membrane, since no raft perturbation was observed on CB2 binding and signalling after the disruption of lipid rafts by cholesterol depletion with methyl-beta-cyclodextrin (MCD) [76].

The activation of cannabinoid receptors by their agonists initiates the transduction of cannabinoid signalling. The stimulation of CB receptors triggers Gi/0 proteins [77, 78] leading to the initiation of several cellular mechanisms that include adenylyl cyclase (AC) inhibition, mitogen-activated protein kinases (MAPKs) activation, modulation of ion channels by activation of inward rectifying K^+ channels or inhibition of voltage-gated Ca^{2+} channels [79] **(figure 8).** CB2 receptors do not seem to have a role in the modulation of ionic channels [78]. Under Gi/0 inhibition, CB1 but not CB2 may interact with Gs [80] and Gq/11 [81] proteins and consequently lead to different cellular effects.

Inhibition of AC results in decrease of cAMP levels and, because cAMP is a second messenger that stimulates the activity of Protein Kinase A (PKA), the activity of this protein decreases [82-84]. However, the isoform of AC expressed in target cells influences the response of CB receptors activation – inhibition (AC-I, III, V, VI and VIII) or stimulation (AC-II, IV and VII) [85].

MAPK pathways regulate cellular functions such as proliferation, differentiation and apoptosis. The activation of p42/44 MAPKs, also known as Extracellular regulated kinase 1/2 (ERK1/2), by CB agonists is dependent on Gi/0-coupling and requires the recruitment of phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB or Akt) [86]. CB1 can also activate p38 MAPK and c-Jun N-Terminal Kinases (JNK1, JNK2) [87].

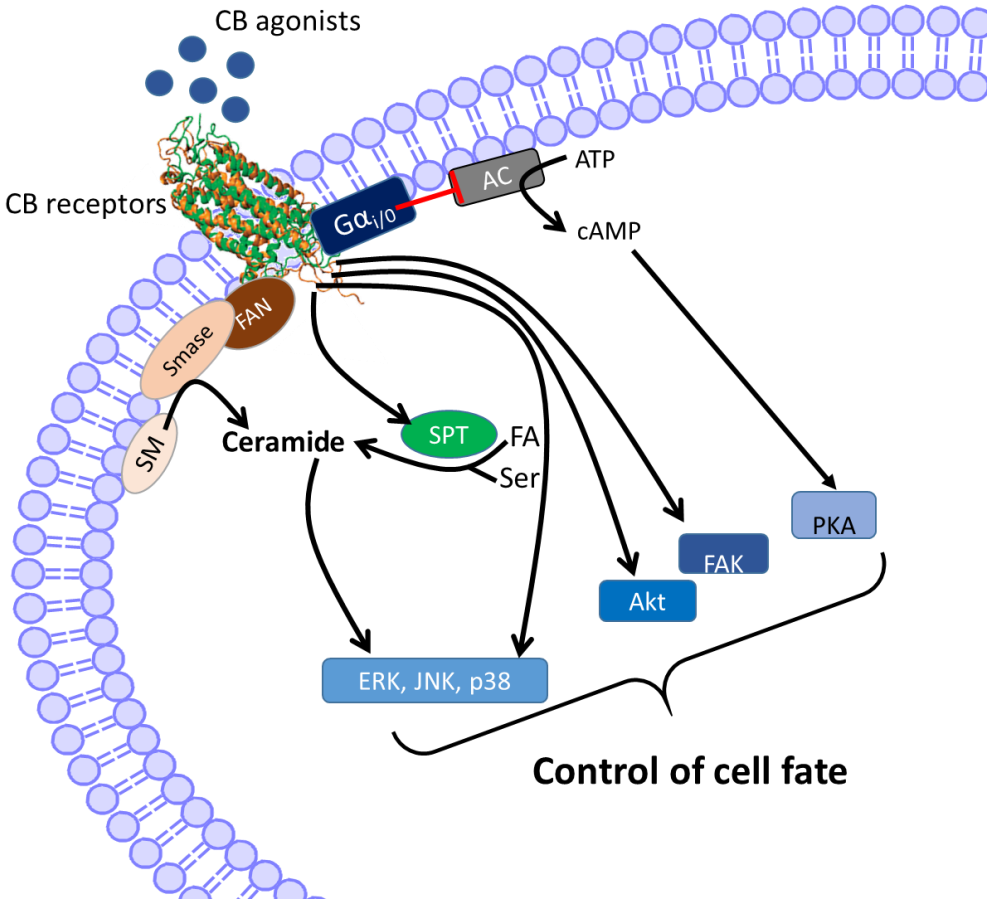

Figure 8 - Cannabinoid signalling pathways after activation of CB receptors coupled to G protein. The inhibition of AC induces a decrease in cAMP levels, leading to a decrease in PKA activity. Activation of CB1 also mediates the stimulation of Akt and FAK. The binding of the adaptor protein FAN to CB1 induces ceramide accumulation through the stimulation of SM hydrolysis by Smase. *De novo* synthesis of ceramide may also occur by SPT, from FA and Ser. As a consequence, ceramide stimulate ERK. JNK and p38. AC- Adenylyl Cyclase; PKA- Protein Kinase A; SM- Sphingomyelin; Smase- Sphingomyelinase; FAN- Factor associated with neutral sphingomyelinase activation; SPT- Serinoylpalmitoyltransferase; Ser- Serine; FA- Fatty acid; ERK- Extracellular signal-regulated kinase; JNK-c-Jun N-terminal kinases; p38- p38 Mitogen-activated protein kinase; FAK- Focal adhesion kinase.

The adaptor protein factor associated with neutral sphingomyelinase activation (FAN), interacting with CB1, mediates the interaction of this receptor and sphingomyelinase (SMase), the enzyme that hydrolysis sphingosine to ceramide, inducing an acute generation of this second messenger. This short-time production of ceramide is related with regulation of metabolic functions and occurs via ERK cascade. The stimulation of *de novo* synthesis of ceramide by serine palmitoyltransferase justifies a long term ceramide accumulation as

a consequence of the activation of CB1. Such occurrence is responsible for ceramide-mediated apoptosis, a process where Raf1/MEK/ERK cascade has an important role [88].

Activation of CB1 also mediates the stimulation of Akt and Focal Adhesion Kinase (FAK). The protein FAK is required during development, since it participates in focal adhesion dynamics between cells, promoting cell migration. It may play other roles in the cell, including the regulation of the tumor suppressor p53 [89]. Akt has a key role in multiple cellular processes such as glucose metabolism, apoptosis, autophagy, cell proliferation, transcription and cell migration.

One of the the most exciting and promising areas of cannabinoid research is the ability of these compounds to control the cell survival/death decision, through the induction/arrest of proliferation, autophagy and/or apoptosis, being these effects dependent on factors, like concentration, timing of drug delivery and type of cells [90].

2.2 Cannabinoids and apoptosis

2.2.1 Apoptosis

Apoptosis is a programmed cell death in which cells undergo a coordinated dismantling of intracellular components without causing damage to the neighbor cells and inflammation. This process is involved in various physiological processes including normal cell turnover, proper development and functioning of the immune system, embryonic development and chemical-induced cell death. Abnormal apoptosis, either diminished or excessive, is associated with some disease conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer [91]. Thus, the control of the apoptotic-signaling pathways is recognized for its immense therapeutic potential and justifies the study of compounds that are able to modulate apoptosis, like cannabinoids.

Caspases are cysteine-proteases essential in apoptosis [92]. Their inactive zymogens gain catalytic activity following apoptotic signals. The caspases involved in apoptosis have been classified as initiator caspases (caspase-8, -9 and -10) and executioner caspases (caspase-3, -6 and -7). Initiator caspases activate executioner caspases that subsequently lead to the generation of a cascade of signaling events for the controlled degradation of key structural proteins and cellular components. This, results in the morphological hallmarks of

apoptosis such as DNA fragmentation, extensive membrane blebbing and separation of the fragments into apoptotic bodies [93].

Two main apoptotic pathways, recruiting different initiator caspases can be distinguished: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway **(figure 9)**. Both pathways converge into the execution phase, the final stage of apoptosis, started by the activation of the execution caspases -3, -6 and -7 with subsequent cleavage of various substrates including cytokeratins, poly (ADP-ribose) polymerase (PARP), the plasma membrane cytoskeletal protein alpha fodrin, the nuclear mitotic apparatus protein (NuMA) and others [94]. Caspase-3 is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) and specifically activates the endonuclease caspase-activated DNase (CAD), by cleavage of the inhibitor of caspase activated DNase (ICAD) [95]. CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies [96], that undergo phagocytic uptake. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells is the hallmark of this phase [97].

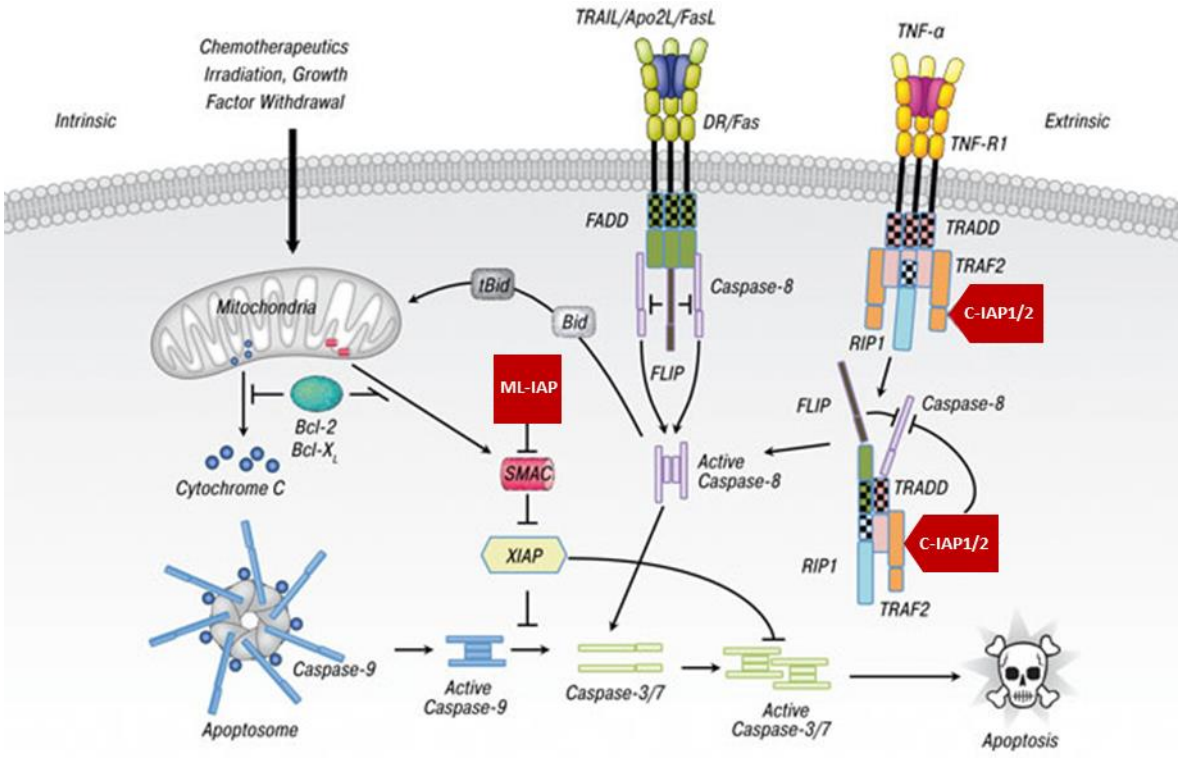

Figure 9 - Intrinsic and extrinsic apoptotic pathways, from [98]

The extrinsic pathway involves transmembrane receptor-mediated interactions. The death receptors are members of the tumor necrosis factor (TNF) receptor superfamily, which have death domains [99]. Upon ligand binding, cytoplasmic adapter proteins that exhibit corresponding death domains are recruited. The binding of Fas ligand to Fas receptor results in the binding of the Fas-associated protein with death domain (FADD), and the binding of TNF ligand to TNF receptor results in the binding of TNF receptor 1-associated protein with death domain (TRADD) with recruitment of FADD and receptor-interacting serine/threonine-protein (RIP) [100, 101]. FADD then associates with procaspase-8 via dimerization of the death effector domain and a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 into active caspase-8 [102]. At this stage, either apoptosis is executed or FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) can bind to FADD and caspase-8, inhibiting death receptor mediated apoptosis [103].

The mitochondrial pathway can be activated by signals involving growth factors, hormones and cytokines, radiation, toxins, hypoxia, hyperthermia, viral infections, oxidative and reticular stress. All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition pore (MPT), loss of the mitochondrial membrane potential ($\Delta\psi m$) and release of two main groups of pro-apoptotic proteins, (i) cytochrome *c*, Smac/DIABLO and the serine protease HtrA2/Omi and (ii) apoptosis inducing factor (AIF), endonuclease G and CAD, from the intermembrane space into the cytosol [104]. Cytochrome *c* binds and activates the apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9, forming an “apoptosome” [105]. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAPs (inhibitors of apoptosis proteins) [106]. AIF and Endonuclease G, which function in a caspase-independent manner, translocate to the nucleus and cause cleavage of nuclear chromatin [107, 108]. CAD is subsequently released from the mitochondria and translocate to the nucleus after cleavage by caspase-3, leading to DNA fragmentation and a more pronounced and advanced chromatin condensation [109]. The control and regulation of these apoptotic mitochondrial events occurs through different members of the Bcl-2 family of proteins [110]. The Bcl-2 family of proteins control mitochondrial membrane permeability and can be either pro-apoptotic (Bax, Bak, Bid, Bad, Bim and Bik) or anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS and Bcl-w). It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome *c* release from the mitochondria via alteration of mitochondrial membrane permeability. Bad can heterodimerize with Bcl-Xl or

Bcl-2, neutralizing their protective effect of inhibition of the release of cytochrome *c* from the mitochondria, promoting cell death.

The “cross-talk” between the extrinsic and the intrinsic pathways may also occur. For example, the mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase-8 cleavage of Bid. Then, cleaved-Bid migrates to mitochondria where it induces permeabilization of the outer mitochondrial membrane that is dependent on the pro-apoptotic proteins Bax and/or Bak, and thus Bid acts as a sentinel for the cooperation of both apoptotic pathways [111].

2.2.2 Cannabinoid-induced apoptosis

A growing number of papers are reporting cannabinoid-induced apoptosis in several systems. Cannabinoids have been shown to induce apoptotic cell death of C6 glioma cells [112], PC-12 pheochromocytoma cells [113], CHP100 human neuroblastoma cells [114] and hippocampal neurons [115]. Moreover, *in vivo* studies performed in rats bearing malignant gliomas showed that THC and WIN treatments resulted in an increase in time of survival or eradication of the tumors, compared to untreated animals [116]. The implication of CB1 receptor was showed in several cells (glioma C6, pheochromocytoma PC-12 and hippocampal neurons) and, in the case of C6 glioma cells, the involvement of CB2 was also observed [116]. Moreover, vanilloid receptors seem to be involved in AEA-induced apoptosis of CHP100 neuroblastoma cells [114]. In the PC-12 cells, AEA induced superoxide generation that culminate in caspase-3 activation, leading to apoptosis [113].

The apoptotic death of glioma cells seems to be dependent on the generation of ceramide [117]. In fact, it has been suggested that cannabinoids enhance ceramide synthesis *de novo* via induction of serinoylpalmitoyltransferase (SPT) [118]. In other systems, pharmacological inhibition of *de novo* ceramide synthesis also prevents cannabinoid-induced death, as in the case of prostate tumor cells [119].

Synthetic cannabinoids may induce apoptosis through a caspase-3-dependent mechanism, as observed in the mouse forebrain cultures. Moreover, the results indicate that the cytotoxicity of synthetic cannabinoids towards primary neuronal cells is CB1-mediated [120]. In the case of EL-7402 human hepatocellular carcinoma cells, WIN-induced apoptosis is executed via up-regulation of Bax and down-regulation of Bcl-2 expression, alterations in the mitochondrial membrane potential and increase of caspase-3, -8 and -9 activities [121].

Anandamide, in tumorigenic keratinocytes that overexpress COX-2, activated the PKR-like ER kinase (PERK), inositol requiring kinase-1 (IRE1), the transcription factor-6 (ATF6) ER-stress pathways and the ER-stress apoptosis-associated proteins, C/EBP homologous protein-10 (CHOP10), caspase-12, and caspase-3. [122]. The same eCB induced cytotoxicity against human melanoma cells through a complex mechanism, which involves COX-2 and LOX-derived products, CB1 activation and lipid raft modulation [123].

In the case of colorectal cancer cells, cannabigerol (CBG), a safe non-psychotropic cannabis-derived cannabinoid, promoted apoptosis, stimulated ROS production, upregulated CHOP mRNA and reduced cell growth. In addition, *in vivo*, CBG inhibited the growth of xenograft tumours as well as chemically induced colon carcinogenesis [124]. Moreover, a study made in multiple myeloma, showed that cannabidiol (CBD) by itself or in synergy with the proteasome inhibitor bortezomib (BORT), a commonly used compound in the treatment of this malignancy, strongly inhibited growth, arrested cell cycle progression and induced cell death by regulating the ERK, AKT and NF-κB pathways [125]. Similarly, in chondrocytes, AEA induced activation of caspase-3, -8, and -9, FAK cleavage, inhibition of AKT activation, sustained activation of ERK, JNK, and p38 [126].

In the reproductive system, AEA induced apoptosis in decidual cells through the involvement of ceramide synthesis, p38 activation and mitochondrial disfunction [127]. In addition, this eCB also induced apoptosis in human cytrophoblasts and in BeWo cells [128]. Moreover, an apoptotic cell death of BeWo cells was reported after 2-AG treatment in BeWo cells through the induction of caspase-3/7 and -9 activities, loss of Δψm and increase in ROS generation [129].

Nevertheless, further investigation is necessary to recognize the specific downstream targets involved in cannabinoid-induced apoptosis and of the relative contribution that the apoptotic mechanisms may have *in vivo*.

2.3 Cannabinoids and autophagy

Parts of this point are transcribed from the manuscript *"Cannabinoid-induced autophagy: protective or death role?"*, which full version is included in appendix.

2.3.1 Autophagy

Autophagy is an evolutionary conserved catabolic process that targets portions of cytoplasm, damaged organelles and proteins for lysosome degradation and it has crucial roles in development and disease. In fact, basal autophagy is normal and necessary to remove aged and damaged organelles and proteins, thereby acting as a constitutive quality control mechanism [130]. It also allows cell-survival in low-nutrient conditions, playing a cytoprotective role. This process occurs naturally in the organism [131-133], though when excessive or uncontrolled, autophagy is able to induce cell death, process referred to as a "type II programmed cell death", as opposed to "type I programmed cell death" or apoptosis [134, 135]. Even in this case, it depends on cell stimuli, such as endoplasmic reticulum (ER) stress, genotoxic/ oncogenic stress, compromised apoptosis, growth factor deprivation, radiation and/ or drugs.

There are two main non-selective canonical pathways to reach autophagy: (i) microautophagy and (ii) macroautophagy (or just autophagy). Microautophagy involves the invagination of the lysosomal membrane, with incorporation and digestion of cytosolic proteins and organelles by lysosomes, without the formation of autophagosomes. It ensures the turnover of long-lived proteins in basal conditions [136, 137]. On the other hand, macroautophagy is responsible for the degradation of soluble proteins and organelles, under stress conditions, by the formation of double-membrane vacuoles named autophagosomes, which sequester portions of the cytoplasm, that after a fusion with lysosomes and the formation of the autolysosome, are digested by lysosomal hydrolases [137-139] **(Figure 10).** Other types of selective autophagy recognize and select for degradation of the cargo (subcellular structures), such as mitochondria, peroxisomes, ribosomes and protein aggregates. These specific types of autophagy are called mitophagy [140, 141], macropexophagy [142, 143] , and chaperone-mediated autophagy [144, 145]. Recent studies have elucidated a novel evolutionarily conserved receptor-mediated selective autophagy, "ER-phagy", that regulates the turnover, function and structure of the ER [146, 147].

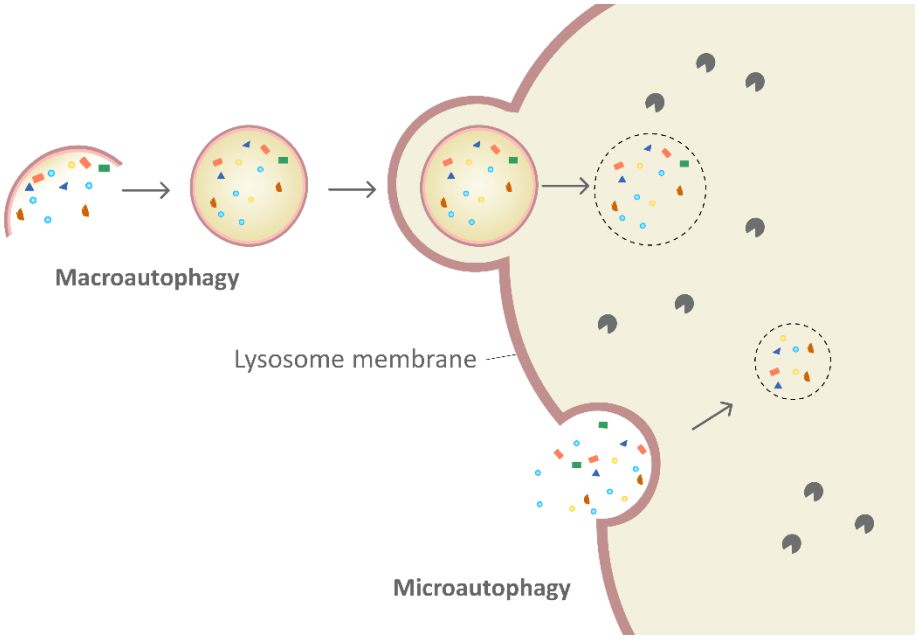

Figure 10 - Schematic illustration showing the two main types of autophagy. Macroautophagy, with the formation of the autophagosome and microautophagy, involving invagination of lysosome membrane.

The autophagic process was first visualized and studied in yeast. This highly organized intralysosomal degradation pathway is regulated by a large family of genes, the autophagy-related (atg) genes [148-151], which play essential roles at different stages of the autophagic process, including induction, vesicle formation and autophagosome degradation [152]. Upon autophagic stimulus, autophagy starts with the formation of the pre-autophagosome structure [153, 154], that consists of three steps: nucleation, elongation and maturation of the phagophore (a membrane derived from ER, trans-Golgi or endosomes) [155-159]. The phagophore is characterized by transient recruitment of the Atg proteins, and its nucleation requires phosphatidylinositol 3-kinase catalytic subunit type III (PI3K III) activation [155] and the formation of a complex with Beclin-1 [160, 161]. The elongation process leads to the formation of an Atg12–Atg5–Atg16 complex, the lipidation of the microtubule-associated protein 1A/1B-light chain 3 (LC3-I) and its conversion into an LC3-phosphatidylethanolamine conjugate (LC3-II), which acts as a structural component inserted in the double-membrane, associated with mature autophagosomes [155, 160, 162-164]. Upon maturation, the autophagosome fuses with late endosomes or lysosomes to form the autolysosome, and its content is digested via the endosomal-lysosomal system [162, 165] **(Figure 11).**

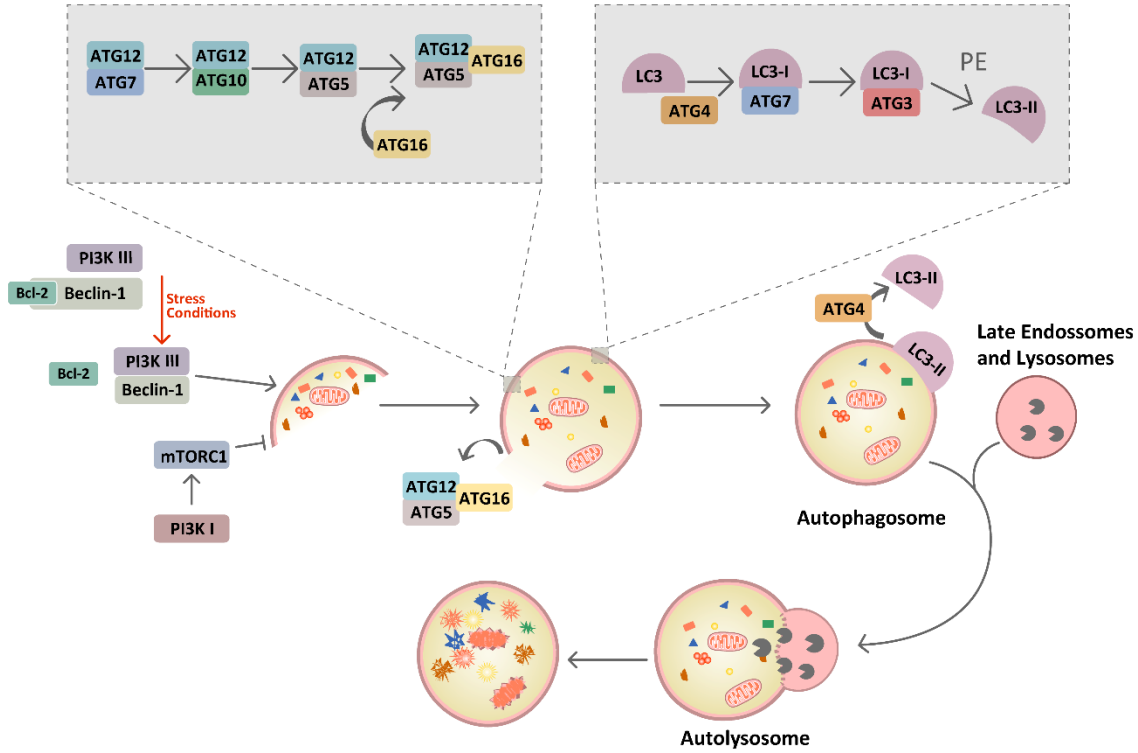

Figure 11 - Schematic depiction of the molecular mechanism of autophagy. Upon autophagic stimulus, autophagy starts with the formation of the pre-autophagosome structure, which consists of three steps: nucleation, elongation and maturation of the phagophore. Phagophore formation is stimulated by the formation of the PI3KIII/Beclin-1 complex and inhibition of mTORC1. For elongation, the Atg5–Atg12-Atg16 conjugation and multimerization of the phagophore occurs. In addition, LC3 processing and insertion into the extending phagophore membrane also occurs. Then, the maturation of the autophagosome is accompanied by recycling of LC3-II by Atg4 and release of the Atg5–Atg12-Atg16 complex. This is followed by fusion of the autophagosome with the late endosomes and lysosomes and proteolytic degradation of the engulfed molecules.

Mammalian Beclin-1 is also an important protein for the autophagic process, that acts as a platform for the recruitment and activation of the PI3K III complex, essential for the initiation of autophagy [161, 162]. Under non-stress conditions, Beclin-1 interacts with the Bcl-2 homology domains (BH3) of the anti-apoptotic proteins of the Bcl-2 family to inhibit the formation of the Beclin-1/PI3K III complex, a mechanism that prevents activation of autophagy [152, 166] [167]. Conversely, the dissociation of Bcl-2 or Bcl-xL from Beclin-1 activates autophagy in response to nutrient deprivation and other physiological stimuli [152]. In addition, the UV radiation resistance-associated gene (UVRAG) [168], the Bax-interacting factor 1 (Bif-1) [169] and the activating molecule in BECN1-regulated autophagy protein 1 (Ambra-1) [170], are activated upon Bcl-2 release from the Beclin-1 complex, acting as positive regulators of the interaction between Beclin-1 with PI3K III [162]. Phosphatidylinositol 3-kinase catalytic subunit type 1 (PI3K I) is also an important regulator

of the first steps of autophagy. It interacts with kinases inducing the phosphorylation of the Protein kinase B (Akt). Upon its activation this kinase phosphorylates TSC1/TSC2 protein complex that become inactive and unable to inactivate the G protein Rheb. The active form of Rheb further activates the mammalian target of the rapamycin complex 1 (mTORC1), thus inhibiting autophagy [155, 171].

In mammalian cells, the protein complex mTORC1 acts as a nutrient/energy/redox sensor. This protein complex is formed by the protein mTOR itself and regulatory-associated proteins [165, 172]. When nutrients and energy are prevalent, mTOR hyper-phosphorylates Unc-51-Like Kinase (ULK), inhibits the formation of the autophagic-induction complex, working as a repressor of autophagy [173]. However, under starvation conditions, the inhibitory activity of mTOR is rapidly shut down [162]. Moreover, mTOR integrates signals that either inhibit autophagy via PI3K/Akt or trigger autophagy via activation of adenosine monophosphate-activated protein kinase (AMPK) [165, 174] . Thus, the key regulators of mTOR are Akt and AMPK. The kinase Akt is stimulated by growth factors to activate mTOR and inhibit autophagy [175], while AMPK is a kinase that is activated in response to an energy deficit, with an increased AMP/ATP ratio, increased cytosolic Ca^{2+} concentration or genotoxic stress [176]. Thus, downregulation of Akt is associated with low levels of active mTOR, allowing autophagy to occur, whereas AMPK activation represses mTOR and, thus, promotes autophagy [155, 165, 177, 178].

Autophagy has been demonstrated to be relevant for processes as diverse as neurodegeneration, immune function, cancer, ageing and development [152, 179]. Recent studies have shown that its role in cell survival represents only a part of its function, since autophagy may also contribute to cell death.

2.3.2 Cannabinoid-induced autophagy

The majority of studies about cannabinoid-induced autophagy have been performed in tumor models. It has been demonstrated that cannabinoids induce autophagy in various types of cancer cell lines, and that, in most cases, this antineoplastic activity is counteracted by the inhibition (pharmacological or genetic) of autophagy, suggesting that this process is required for the cannabinoid's antiproliferative action. On the other hand, some studies have also found evidence of autophagy in non-tumor cells.

It has been demonstrated that CB1 receptor activation affects lysosomal activity and degradation of damaged macromolecules. This suggests that it may influence the course and onset of brain aging [180]. On the other hand, the activation of CB2 by the synthetic cannabinoid HU-308 can alleviate pathogenesis of experimental autoimmune encephalomyelitis via activation of autophagy [181]. Moreover, Sativex® reduces tau and amyloid deposition in the hippocampus and cerebral cortex, in a mouse model of complex neurodegenerative disorders, through the reduction of free radicals, enhancement of mitochondrial activity and stimulation of autophagy [182]. Besides its action in neurodegenerative diseases, CBD can protect mouse liver cells from acute alcohol-induced steatosis through multiple mechanisms including attenuation of alcohol-mediated oxidative stress, prevention of JNK/MAPK (c-Jun N-terminal kinase/ mitogen-activated protein kinases) activation and increase of autophagy [183]. In reproductive processes, the synthetic cannabinoid methanandamide induced, *in vivo* or *in vitro*, an autophagic response in pre-implantation mouse embryos [184].

A recent study has shown that CBD, arachidonyl-2'-chloroethylamide (ACEA) and AEA induce autophagy in a dose-dependent manner in fully differentiated Caco-2 cells. ACEA and AEA induced canonical autophagy through CB1 activation, whereas CBD did not require the entire set of Atg proteins and was CB1-independent [185, 186].

Recent studies carried out in different experimental tumor models have reported activation of autophagy as a consequence of cannabinoid treatment. The way that cannabinoids interact with cancer cells was found to depend on the tumor system, including the type of receptors to which they bind and the consequent activated pathways **(Figure 12).**

Treatment with phytocannabinoids seems to cause an autophagic cell death, in which autophagy precedes apoptosis. Salazar et al. (2009) have shown that THC is able to induce autophagy-mediated apoptotic cell death. This anti-tumoral action relies, at least in part, on the upregulation of several ER stress-related proteins, including TRIB3, allowing autophagy to occur [187]. Furthermore, the authors found that THC also induced accumulation of *de novo*-synthesis of ceramide, upregulation of stress-regulated protein p8 and of its downstream targets, activating transcription factor 4 (ATF4), CHOP and TRIB3. This supports the involvement of ceramide accumulation in cannabinoid-triggered ER stress and autophagy.

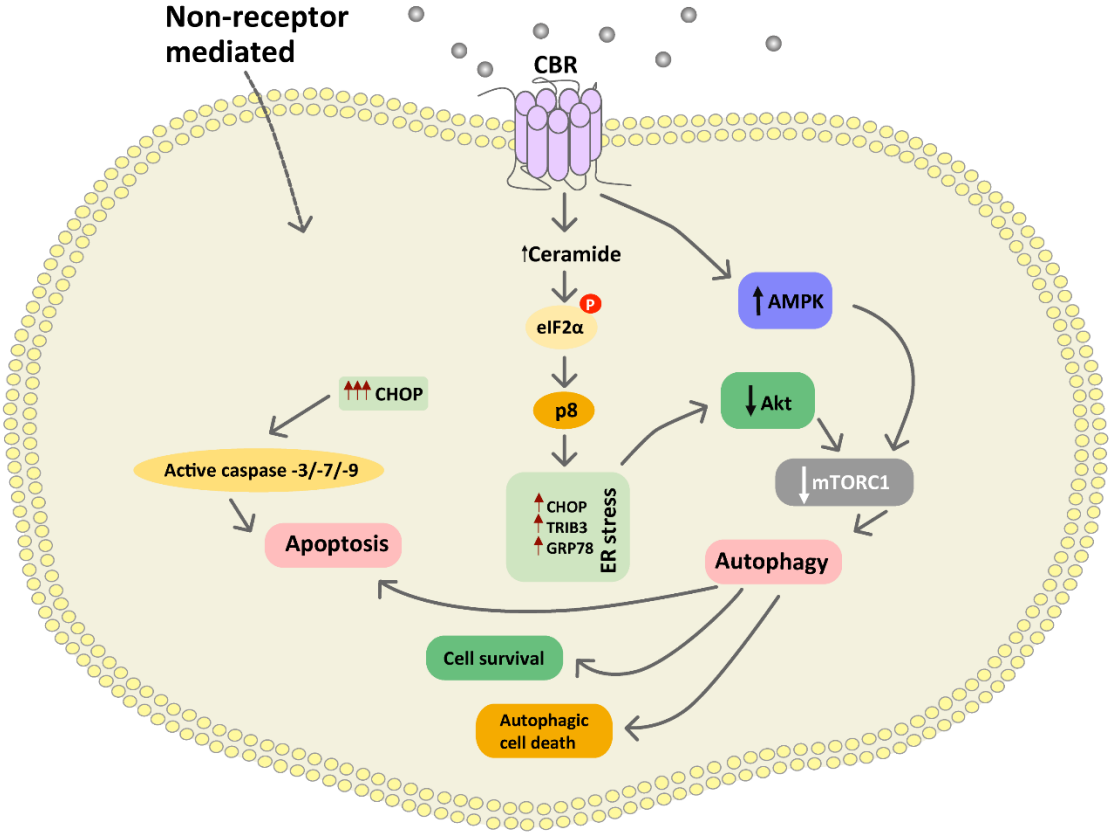

Figure 12 - A schematic representation of the proposed mechanisms by which cannabinoids induce autophagy, in an independent or receptor-dependent manner. Cannabinoid treatment stimulates autophagy mainly via two different mechanisms: activation of AMPK or stimulation of de novo synthesis of ceramide, which leads to an ER stress response with an increase in eIF2α phosphorylation, upregulation of p8 and its downstream targets such as ATF4, CHOP, TRIB3, GRP78 and subsequent inhibition of the Akt/mTORC1 axis. When the increased level in CHOP is not accompanied by the increase in GRP78 or TRIB3, it leads to a mitochondrial-dependent apoptosis. Stimulation of autophagy by cannabinoids may lead to cell survival, cell death or apoptosis.

A study in glioma cells has described the gene expression profile characteristic of THC-resistant cells, being the growth factor Mdk (Midkine, also known as neurite growth-promoting factor 2 - NEGF2), the most strongly associated gene. This growth factor has been associated with modulation of proliferation and migration of various types of cancer cells and is often overexpressed in malignant tumors, acting as a protective factor against cell death [188, 189]. Moreover, a similar resistance mechanism was found to operate in pancreatic cancer cells. The cannabinoid-resistant pancreatic cancer cell line PANC-1 exhibited higher levels of Mdk than the cannabinoid-sensitive pancreatic cancer cell line MIA PaCa-2. Mdk silence sensitized cannabinoid-resistant cell lines as well as a primary culture of glioma cells to THC-induced autophagy and apoptosis [190].

In breast cancer cells, treatment with CBD induced ER stress and subsequently increased phosphorylation of eukaryotic translation initiation factor 2, subunit 1 alpha (eIF2α) and decreased phosphorylated mTORC1 levels. The increase in the autophagic marker LC3-II was accompanied by an increase in cleaved-PARP (Poly (ADP ribose) polymerase 1), indicating a cross-talk between autophagy and apoptosis. The authors found that Beclin-1 plays a central role in the induction of CBD-mediated apoptosis since, in addition to enhancing the interaction between Beclin-1 and PI3K III, CBD inhibited the association between Beclin-1 and Bcl-2. The effects were receptor-independent, causing ROS generation, translocation of BID to the mitochondria and activation of the intrinsic apoptotic pathway. When the increase of ROS generation is blocked, the apoptosis and autophagy processes are prevented. [191]. Very recently it was shown that this cannabinoid triggers glioma stem-like cells (GSCs) differentiation by inducing an autophagic process, through activation of the transient receptor potential vanilloid 2 (TRPV2). The autophagy promoting activity of CBD seemed to be regulated by the AKT/mTOR protein kinase network and PTEN could be involved. GSCs are a subpopulation believed to be involved in glioblastoma multiforme tumor initiation and acquired chemoresistance. So, CBD-induced autophagy acts as a tumor suppressing mechanism. [192]. A study carried out by Armstrong et al, using THC and CBD (Sativex®) showed that these cannabinoids led melanoma cells to a non-canonical autophagic-mediated apoptotic cell death, inhibiting melanoma viability, proliferation and tumor growth. The referred study suggest that cannabinoid treatment may be of clinical benefit for metastatic melanoma [193].

CB2 receptor activation was indicated as being responsible for the antitumorigenic effects of THC and JWH-015, a synthetic cannabinoid, in hepatocellular carcinoma (HCC) cell lines and in human HCC xenografts. The TRIB3/Akt/mTORC1 pathway appears to be involved. In addition, increased phosphorylation of AMPK seems to be necessary for the autophagy-mediated cell death by cannabinoids in HCC cells [194]. More recently, it was also shown that pharmacological inhibition of PPAR-γ decreased the death effects of cannabinoids in HCC cells. Further, downregulation of TRIB3 reduced the expression of PPAR-γ and induced p62 accumulation [195].

Pellerito and his group (2014) have also reported ER stress induction and cell death after WIN55,212-2, treatment in colon cancer cell lines. In accordance with the previously described studies, the levels of phosphorylated Akt decreased and the levels of TRIB3 and CHOP increased. By contrast, Beclin-1 knockdown was ineffective in counteracting these effects, confirming that WIN induced a Beclin-1 independent autophagic pathway. In this study, however, the involvement of an autophagic process appears to play a pro-survival

role against the cytotoxic effects of the drug. Such data provided evidence for a role for PPAR-γ in cannabinoid signaling, being the expression of this receptor significantly down-regulated after WIN treatment [196].

A recent work performed by the same group has studied the effect of this cannabinoid on human osteosarcoma cells. WIN treatment induced G_2/M cell cycle arrest, which was associated with the induction of the main markers of ER stress, such as CHOP, TRIB3 and GRP78 and enhancement of LC3-II, indicating an autophagic response. In correlation with the preceding study, no alteration in Beclin-1 levels were observed [197].

Due to its antiproliferative effects, cannabinoids are being combined with other existing anti-cancer therapies such as temozolomide (TMZ) [198], TNF-alpha-related apoptosis-inducing ligand (TRAIL) [197], gemcitabine (GEM) [199] and radiation [200]. These studies are of particular relevance to patients using cannabinoid-based drugs to improve cancer therapies.

3. Cannabinoids and pregnancy

3.1 The human placenta and trophoblast cells

The placenta is a fascinating organ, shared by the mother and the growing fetus in which several well balanced phenomena such as cell proliferation, differentiation, invasion and apoptosis co-exist [201, 202]. Serving as the interface between the fetal and maternal environments, the human placenta is structured as a villous tree and is in direct contact with the maternal blood and thus referred to as hemochorial [203]. The placenta is involved in the exchange of gases, nutrients and waste products between the mother and the growing fetus. Furthermore, serves as an endocrine organ, producing several pregnancy-associated hormones and growth factors, which affect pregnancy, metabolism, fetal growth, parturition and other functions, and warrants the protection of the fetus from maternal immunity attack [204-206].

Regarding the anatomy (**figure 13)**, the utero-placental unit is composed of both fetal and maternal tissue, derived from the chorionic sac and from the endometrium, respectively. The fetal part, the chorionic plate, carries the fetal chorionic blood vessels, while the maternal part is known as basal plate. The intervillous space comprises the space between the chorionic and basal plate and contain the chorionic villi, within which fetal blood is separated by only three or four cell layers from maternal blood. This intervillous space is completely lined with multinucleated syncytiotrophoblasts, the cells that contact with the maternal blood, which enters this space via spiral endometrial arteries, bathes the villi and drains back through endometrial veins. The fetal blood, which is oxygen-deficient, passes through two umbilical arteries and the branched chorionic arteries to the extensive arterio-capillary-venous system within the chorionic villi. Then, the oxygenated fetal blood returns to the fetus via chorionic veins and umbilical vein [207]. The fetal and maternal side of the human placenta are represented in **figure 14**.

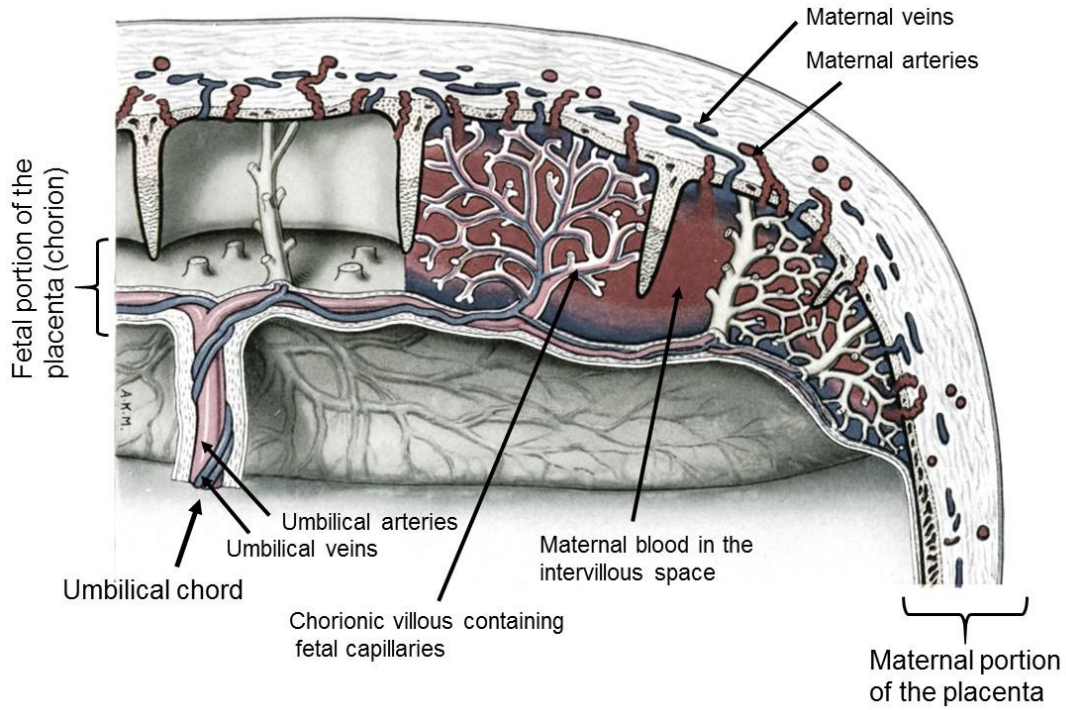

Figure 13 - Human placenta anatomy. Adapted from [208]**.**

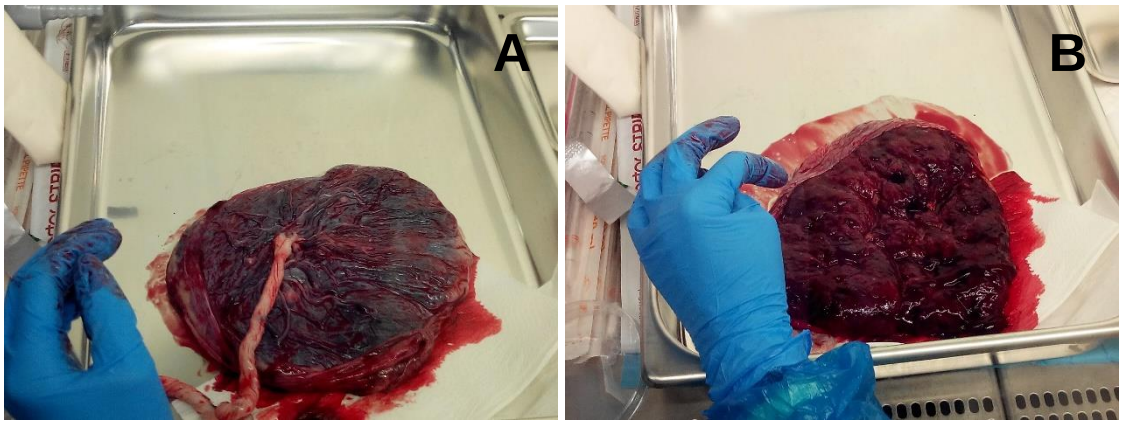

Figure 14 - Human placenta. Fetal (A) and maternal (B) side of human term placenta

Besides being characterized by the direct contact between maternal blood and trophoblast, human placenta is also defined by its highly invasive placentation, reaching deep in the pregnant uterus [203], exhibiting the most pronounced infiltration of the myometrium among all eutherian mammals [209].

The first cell differentiation event in mammalian development occurs in the transition from the morula to the blastocyst state and leads to the establishment of two distinct cell lineages: the trophectoderm (TE) and the inner mass (ICM) **(Figure 15).** The first is the origin of trophoblasts cells, which constitute the epithelial compartment of the placenta; and the second gives rise to the embryo.

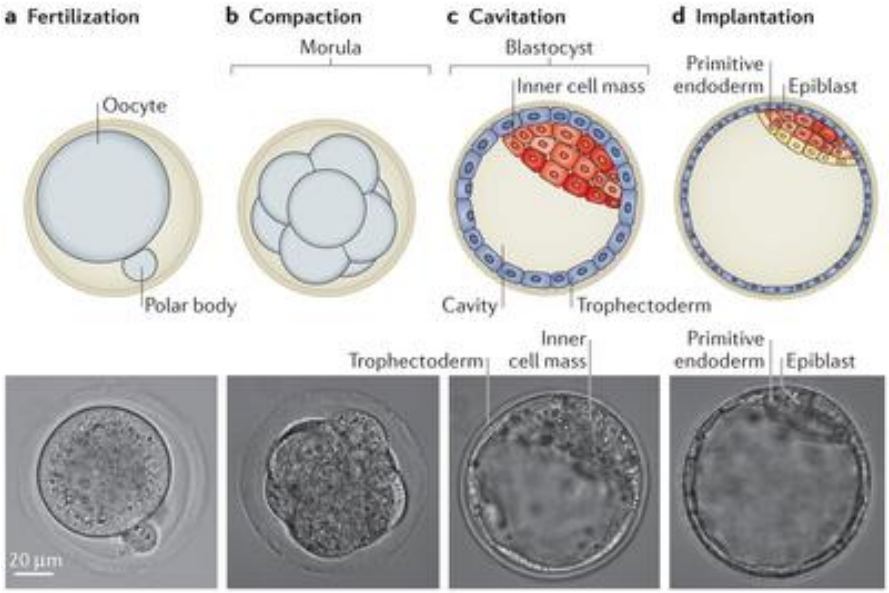

Figure 15 - Pre-implantation development. From [210]

During placentation, trophoblast cells differentiate through two major pathways: the villous and extravillous pathways **(figure 16).** In the villous pathway, cytotrophoblast cells (CTs) are the ones that reside at the basement membrane of placental villi and separate trophoblasts from the underlying mesenchymal villous core, in a continuous turnover. These cells undergo apoptosis, differentiate and fuse to form the multinucleated syncytiotrophoblasts (ST), which surround the placental villous and, as referred, are in direct contact with the maternal blood within the intervillous space **(Figure 17 and 18).** Placental ST are involved in the transport of nutrients and gases from the maternal to the fetal circulation and represent the major endocrine unit of the placenta by secreting numerous pregnancy-maintaining hormones including chorionic gonadotropin (hCG), human placental lactogen (hPL), estrogen and progesterone [211].

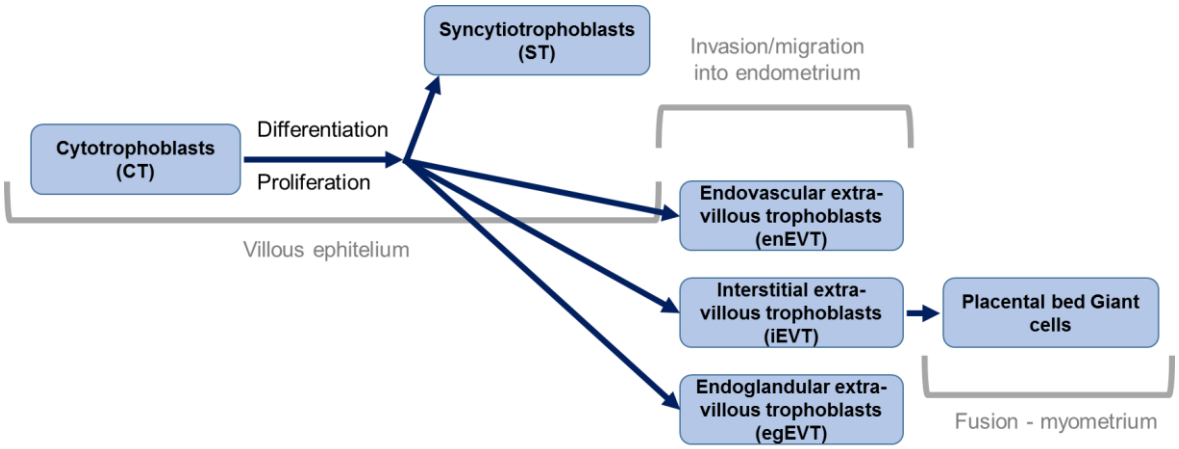

Figure 16 - Trophoblast differentiation and proliferation

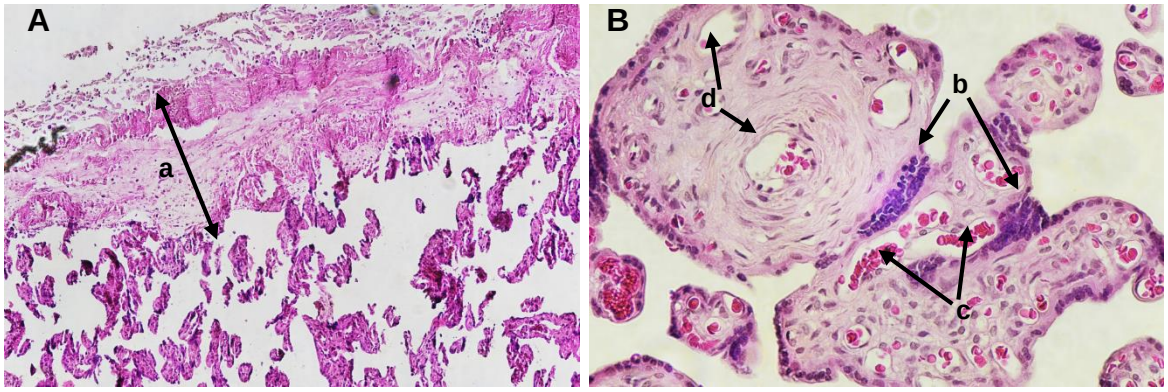

Figure 17 - Histological sections of human placenta stained with haematoxylin and eosin. (A) original magnification 200x; (B) original magnification 400x. Legend: (a) Decidua; (b) Syncytiotrophoblasts; (c) Blood cells; (d) Blood vessels.

In the extravillous pathway, the villous CTs provide a population of progenitor cells that acquire an invasive phenotype and differentiate into either (1) interstitial extravillous trophoblasts (iEVTs), which invade the maternal decidua and a portion of the myometrium, (2) endovascular extravillous trophoblasts (enEVTs), which remodel the maternal vasculature, to guarantee appropriate blood flow to the placenta **(figure 18)**, and (3) endoglandular extravillous trophoblasts (egEVTs), that invade the uterine glands, orient them towards the intervillous space and replace the uterine epithelial cells [203].

Other type of trophoblast cells, the trophoblast giant cells, participate in various processes essential to a successful pregnancy, including blastocyst implantation, remodelling of the maternal decidua, and secretion of hormones that regulate the development of both fetal and maternal compartments of the placenta [212].

These differentiation events are tightly controlled by the interplay of oxygen tension, transcription factors, hormones, growth factors, and other signalling molecules. Due to trophoblast-mediated plugging of the uterine vasculature, early placental development occurs in hypoxic environment, coinciding with a peak in trophoblast prolifferation and placental growth [213]. This may lead to the assumption that hypoxia could be the main trigger of early trophoblast growth and by the end of the first trimester of pregnancy, when blood flow is established, normoxia may trigger trophoblast invasion [214]. Various growth factors, cytokines and chemokines are thought to regulate EVTs function [215]. There are several observations that support the idea that EVTs ability to invade foreign tissue underlies an intrinsic program or at least is not dependent on decidua-specific signals [216, 217]. However, there are also evidences of a pivotal decidua-specific role in the regulation of trophoblast invasion [218]. So, it seems that decidual cells may rather represent a "gatekeeper" function allowing trophoblats to access maternal tissues and blood supply by achieving an accurate balance between under- and over-invasion [203].

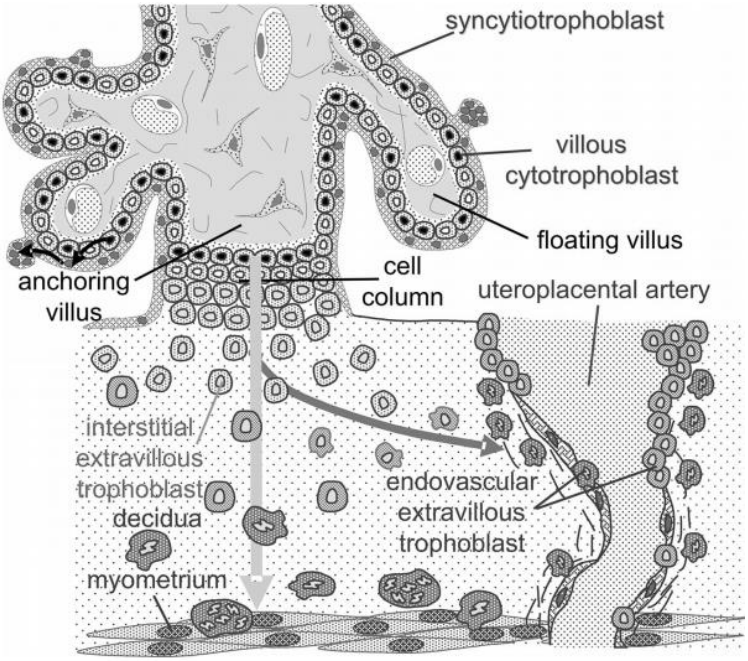

Figure 18 - Placental cytotrophoblasts invasion. From [219]

Abnormal placental development, particularly the inadequate invasion of trophoblast cells into the uterus and the subsequent failure of the remodelling of maternal spiral arteries, with consequent decrease in blood flow to the placenta, is believed to cause pregnancy

disorders like miscarriage, preeclampsia or intrauterine growth restriction [220]. Hence a better knowledge about human trophoblast invasion is mandatory to improve therapeutic intervention.

3.2 Endocannabinoids and pregnancy

Mammalian pregnancy is a complex process, involving oocyte fertilization, a preimplantation phase which includes the embryo development and passage through the oviduct, then implantation in the uterus, and further development supported by a functional placenta. Several studies demonstrate that a tight control of AEA levels are important in several stages of gestation **(figure 19)**. In fact, it has been proposed that AEA is a "guardian angel" or "gatekeeper" of mammalian reproduction [221].

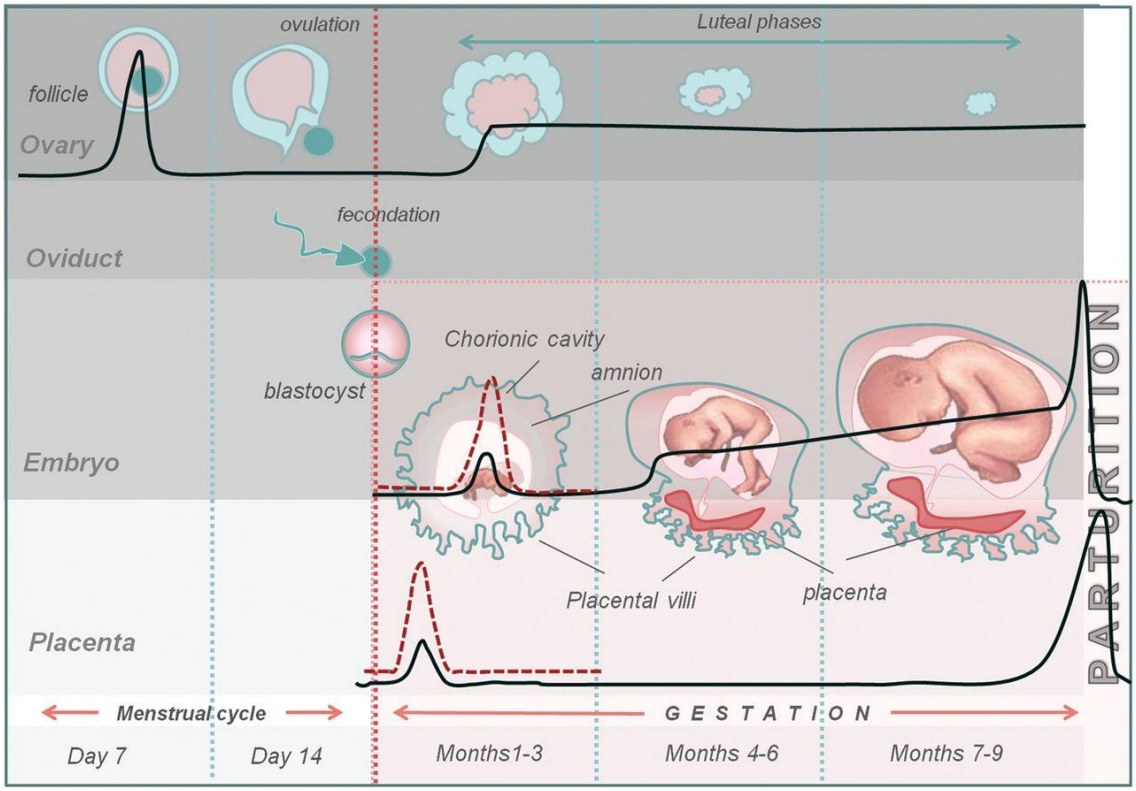

Figure 19 - Levels of anandamide through the menstrual cycle and gestation. Solid black line represent physiological conditions, in which AEA plasma levels are high during follicular phase and lower in the luteal phase of the menstrual cycle, as well at the beginning of implantation. Low AEA levels are necessary to promote uterine receptivity and pregnancy maintenance. At the time of labor, high levels are required for parturition. The red dashed line represent AEA levels under pathological conditions, from placentas of pre-eclampsia patients, ectopic and non-viable pregnancies, as well as lymphocytes from women who miscarry. From [221].

It was suggested that high plasma levels of AEA are required at pre-ovulation [222], whereas low levels are necessary to achieve successful implantation [223]. Several works demonstrate that abnormal cannabinoid signalling, either silenced or enhanced, impairs embryo transport [224]. By using CB1 knockout mice [225, 226], it has been shown that CB1 regulates the oviductal transport of embryos, while CB2 is not involved in this process. In addition, CB1 mRNA has been shown to undergo a temporal variation in human Fallopian tubes, whereas a low expression has been detected in women with ectopic pregnancy, associated with a lower expression in the endometrium [225]. When embryo at the late morula or early blastocyst stage enters in the uterus, and differentiates to become implantation-competent, low levels of AEA in the uterine luminal epithelium are beneficial, because implantation competency requires down-regulation of AEA binding to blastocyst CB1 receptors [227, 228] **(figure 20).** In the uterus, 2-AG showed a similar distribution as AEA during early pregnancy, suggesting that this eCB could also take part in the modulation of implantation [228].

It appears that the positive or negative effect of AEA on reproductive events critically depends on a balance between synthesis and degradation, by NAPE-PLD and FAAH, respectively. High levels of FAAH were also found in the human villous cytotrophoblast [223] and syncytiotrophoblast [229], suggesting that FAAH has a protective role by cleaving AEA and preventing its passage at the maternal-fetal interface.

Evidences of the critical importance of AEA concentrations in implantation were also enhanced in studies on embryos exposed to high levels of AEA, showing embryotoxicity, reduced trophoblast proliferation and implantation failure [224, 230, 231]. Similarly, women undergoing an *in vitro* fertilization program and achieving successful implantation, were found to have low levels of plasma AEA, associated with high levels of FAAH in their peripheral lymphocytes [232, 233].

Studies performed by our group, revealed that cannabinoid signalling plays an important role in several pregnancy events such as decidualization and placentation [234]. The characterization of the ECS in rat haemochorial placenta [235], the spatio-temporal expression patterns of anandamide-binding receptors in rat implantation sites [236], the eCBs levels and expression of their metabolizing enzymes during pregnancy [237] support a role for the ECS during these periods. In addition, for the first time, 2-AG metabolic machinery was characterized in placental trophoblast cells. The results revealed that 2-AG induces a decrease in BeWo cells viability [129] and interferes with human cytotrophoblast cells syncytialization, through a CB receptor-dependent mechanism [238].

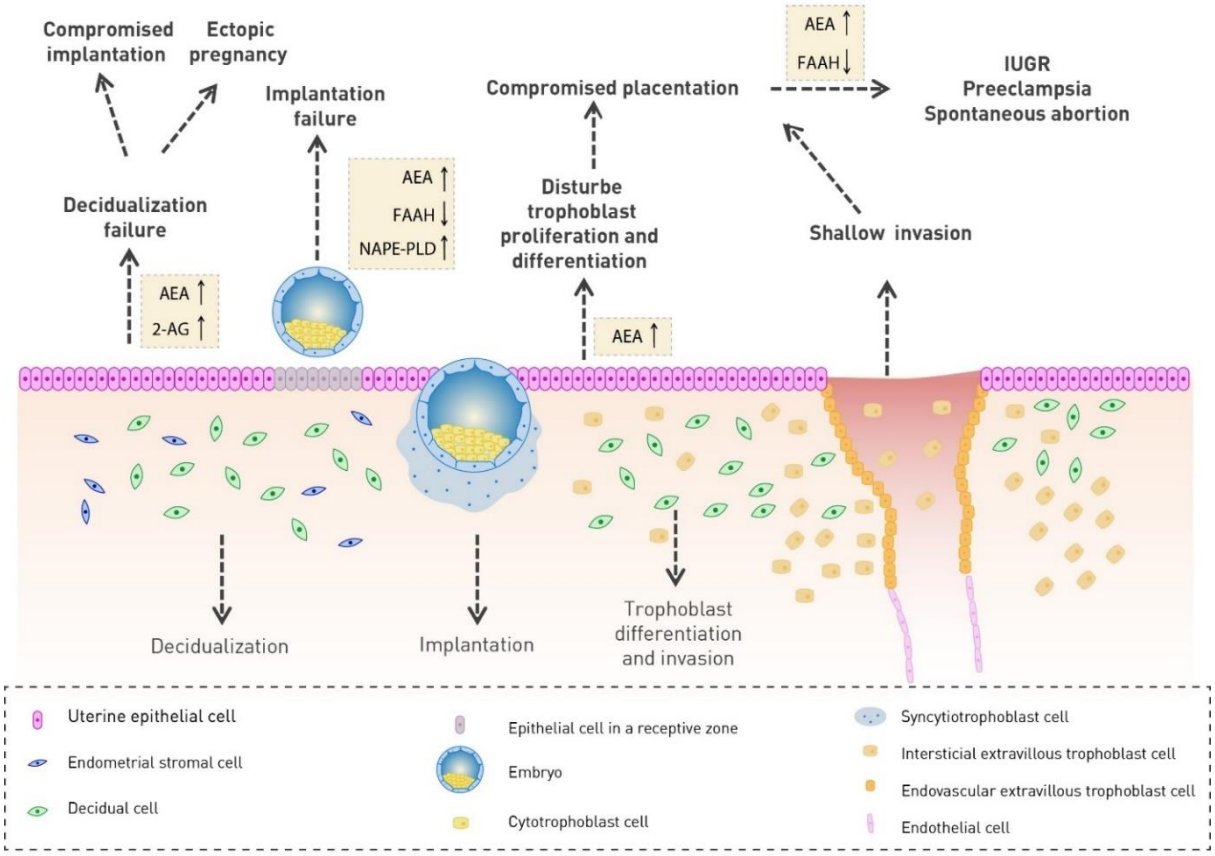

Figure 20 - Potential effects of endocannabinoids at the fetomaternal interface. From [234]

In mouse, cannabinoid signaling via CB1 seems to play a role in parturition [239]. Moreover, it was observed that genetic loss of CB1 leads to preterm delivery and lower birth weight [240]. In humans, prostaglandin production is associated with parturition. Since endocannabinoids significantly increase prostaglandin production in human amnion at term, cannabinoid signaling may play a role in parturition [241]. In fact, AEA plasma levels dramatically increase and are affected by the duration of labor [223].

In summary, it seems that eCBs levels have the potential to direct human fertility towards a positive or negative outcome. Moreover, eCBs levels and/or the activity of their metabolic enzymes in human blood may be considered reliable markers of natural and assisted reproduction [221]. In fact, the reduced level of FAAH in peripheral blood has been recognized as a diagnostic marker of spontaneous miscarriage in healthy women, as well as of failure to achieve an ongoing pregnancy after in vitro fertilization (IVF)-embryo transfer in women undergoing assisted reproduction cycles. In the same context, detection of AEA

in follicular fluid might be a predictor of oocyte maturity [222]. On the other hand, the drop of AEA plasma levels that occurs at ovulation, and less during implantation, could be used as a biomarker for the proper timing of embryo transfer in IVF protocols, and/or in intracytoplasmic sperm injection (ICSI) procedures [233].

3.3 Exocannabinoids and pregnancy

Cannabinoid signaling, as described before, is involved in several stages of pregnancy. So, maternal use of exogenous cannabinoids may disrupt the balance of cannabinoid signaling and compromise pregnancy outcome [242, 243].

Nowadays cannabis preparations are among the illicit drugs most widely used by pregnant women. Indeed, the self-reported use during pregnancy is approximately 2,9% in the US, and reaches 5% in the UK [244]. Chronic exposure to THC impairs women reproductive potential by disrupting the menstrual cycle and suppressing oogenesis. It is also associated with adverse pregnancy outcomes including miscarriage, preterm delivery, intrauterine growth restriction and reduced birth weight [232].

Since the identification of the foetal alcohol syndrome (FAS), there has been an enormous increase in the number of clinical and preclinical reports examining the short- and long-term effects of maternal drug abuse [245]. In recent years, tests carried out in women who regularly use marijuana found trace amounts of THC and cannabidiol (CBD) in their reproductive organs [246]. THC is able to cross the placental barrier during gestation [247] and, due to its high lipophilicity, it can be stored in fat tissue and be transferred to the babies through maternal milk during lactation [248]. Moreover, the slow release of THC from lipid-storage compartments and significant enterohepatic circulation contribute to a long terminal half-life of THC in plasma, in chronic cannabis users [249]. In a pregnant women following heavy, chronic cannabis use, the drug was found in urine testing 3 months after the consumption, proving the very slow release of THC [250]. So, the effects for the foetus are not restricted to the use during pregnancy, but also to the maternal consumption before that.

THC seems to be able to inhibit human trophoblast cells turnover [251], and thus it may interfere with normal placentation. In fact, evidence from a human epidemiological study indicates that regular use of cannabis during pregnancy is associated with decrease in birth weight [8], which is also associated with preterm delivery. In addition, THC abuse during pregnancy may also interfere with synaptogenesis and brain development [252].

Clinical studies reported that children born from women who used cannabis during pregnancy may show some developmental alterations in both childhood (defective memory and poor verbal skills) and adulthood (impulsivity, delinquency and drug addiction) [248, 253, 254].

The effects of maternal use of synthetic cannabinoids during pregnancy is ambiguous due to limited studies in human and a relative short history of the drugs [242]. The possibility that new synthetic cannabinoids adversely impacts pregnancy outcome should not be neglected, since many sCBs have much higher affinity to cannabinoid receptors than the other cannabinoid compounds [255]. During pregnancy, and similar to THC, synthetic cannabinoids, such as WIN, are able to interfere with neuronal processes. Mereu et al., showed in pregnant rats that prenatal exposure to WIN produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release [256]. Later, Castelli et al. showed that adult rats prenatally treated with WIN, presented dysregulation of the ECS, with affected density, affinity and/or function of the cannabinoid receptors, alterations in the AEA levels and activities of the main AEA metabolic enzymes [257]. These findings suggest that prenatal exposure to cannabinoid agonists induces long-term alterations of the ECS in brain areas involved in learning-memory, motor activity and emotional behavior.

Studies on the effects of THC analogues, endocannabinoids and synthetic cannabinoids which act at cannabinoid receptors, may help to better understand the impact of cannabinnoids in pregnancy. Moreover, since the ECS is present and already functional in early pregnancy and is critically involved in crucial steps of pregnancy, the active constituents of cannabis and the synthetic cannabinoids could directly affect this system [242, 245].

4. Aims

The endocannabinoid system is involved in a wide range of physiological and pathological pregnancy-related processes and its components have the potential for being used as biomarkers for reproduction. However, the effects of cannabinoids upon placentation and the implicated mechanisms are still under study. Maternal consumption either by recreational or medicinal use of cannabinoids leads to a placental exposure. This may cause disturbances on the endocannabinoid system homeostasis, ultimately culminating in pregnancy complications. Thus, with this study it is pretended to investigate the influence of different types of cannabinoids in trophoblasts, by using the BeWo cytotrophoblast cell model and primary cultures of cytotrophoblasts and to contribute to the knowledge about the role of the ECS and impact of its deregulation in reproductive events, specifically in placental development.

Chapter II

Material and Methods

1. Materials

Dulbecco's Modified Eagle Medium: fetal bovine serum (FBS), antibiotic–antimycotic solution (penicillin G sodium, streptomycin sulphate and amphotericin B), trypsin, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were from Gibco/Invitrogen Corporation, Carlsbad, CA, USA. Antibody anti-CHOP (GADD 153), β-tubulin and rabbit IgG were from Santa Cruz Biotechnology. Nitrocellulose membranes and Percoll were from GE Healthcare, Chalfont St Giles, Bucks, UK. The Super Signal West Pico Chemiluminescence Detection Kit was from Pierce, Rockford, IL, USA, and X-ray films were from Kodak XAR, Eastman Kodak. Nutrient Mixture F-12 (DMEM/F12), DNAse, paraformaldehyde, Mayer's haematoxylin solution, protease inhibitor cocktail, Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT), Höechst 33342, staurosporine (STS), carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA), H_2O_2, glutathione reductase from baker's yeast, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and Acridine Orange (AO) were from Sigma–Aldrich Co. St. Louis, MO, USA. Ethanol, isopropanol and methanol were from Fisher Scientific, Loughborough, UK. DMSO was from VWR, Fontenay-sous-Bois, France. Z-VAD-FMK was from BD PharMingen, San Diego, CA, USA. Aquamount medium was from BDH Laboratory Supplies, Poole, England. WIN, 2-AG, AM251 and AM630 were from Tocris Bioscience, Bristol, UK. THC was from Lipomed AG, Swiss. CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit and Caspase-Glo 3/7 were from Promega. Giemsa was from Merck. Ninety-six-well white plates were from Thermo Scientific, Roskilde, Denmark. Ninety-six-well black plates were from BD Biosciences, Erembodegem, Belgium.

2. Cell cultures

2.1 BeWo cell line

BeWo is a human choriocarcinoma cell line [258], well-accepted as a cytotrophoblast cell model. This line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

BeWo cells were maintained in culture in DMEM/F12 medium supplemented with 10% (v/v) of FBS and 1% (v/v) of an antibiotic-antimycotic (AB-AM) solution and were incubated at 37°C in 95% air/5% CO_2 humidified atmosphere. Culture medium was changed at every 3 days. After reaching about 80% of confluence, cells were successively sub-cultured to new culture flasks. For this, cells were treated with 0.25% trypsin/EDTA 1 mM for 3 minutes at 37ºC, washed with PBS and collected to centrifuge tubes with culture medium containing 10% FBS (v/v) to inactivate trypsin. Cells were centrifuged at 600 g for 5 minutes at 4ºC. Then cells were counted in a Neubauer chamber and cultured using the cell densities: $1{,}5x10^4$ cells/well (final volume 200 µL) for 96-well plate, $8x10^4$ cells/well (final volume 500 µL) for the 24-well plate and $6x10^5$ (final volume 2000 µL) for the 6-well plate. After adherence (12 hours), the cells were washed with PBS and treated with the compounds, in cell culture medium with 1% (v/v) FBS.

2.2 Isolation and primary cultures of human cytotrophoblasts

Term placentas of normal pregnancies (38-40 weeks of gestation), from Caucasian women living in the Porto region, were immediately collected after spontaneous delivery or elective caesarean section, from Centro Materno-Infantil do Norte - Centro Hospitalar do Porto. All the procedures were conducted in accordance with the Ethical Committee of Centro Hospitalar do Porto, Porto. Cytotrophoblast cells were isolated as described previously [259, 260]. Briefly, placentas were washed with saline solution to remove most of the blood. Then, the villous tissue dissected from at least 10 different regions in the whole placenta. The major blood vessels were discarded by fine dissection. Then, the tissue was subjected to a chemical digestion in a solution of trypsin and DNAseI. Afterwards, the resulting cells were separated in a discontinuous Percoll gradient. After centrifugation (600 g, 10 minutes), the cytotrophoblasts were collected, counted and

seeded with the adequate densities: for the 96-well plates were seeded $1,35x10^5$ cells/well (final volume 200 µL); for the 24-well plates were seeded $1x10^6$ (final volume 500 µL) and for the 6-well plates were seeded $4,5x10^6$ cells/well (final volume 2000 µL).

The cells were incubated in DMEM/F12 medium supplemented with 10% (v/v) of FBS and 1% (v/v) of AB-AM solution at 37°C in 95% air/5% CO_2 humidified atmosphere. After adherence (12 hours), the cells were washed with PBS, incubated with DMEM/F12 medium supplemented with 1% FBS and 1% AB-AM and treated with the compounds, for 24 hours.

3. Cell viability assays

To access cell viability it was used the tetrazolium salt [3-(4,5-dimetylthiazol-2-yl)-2,5-dipheniltetrazolium bromide] (MTT) assay and measured the activity of the enzyme lactate dehydrogenase (LDH) in cell culture medium. For that, BeWo cells were plated in 96-well plates. After 12 hours for adhesion, the medium was replaced with DMEM/F12 medium with 1% FBS and 1% AB-AM solution in the presence or absence of the cannabinoids (1-50 µM), and the cells were incubated for 24 hours. The yellow tetrazole MTT (final concentration: 0,5 mg/mL) was added, and the cells were incubated for 3 hours at 37°C. The formed purple formazan was dissolved in a solution of DMSO:isopropanol (3:1) and spectrophotometrically quantified at 540 nm by using a Multiskan Ascent microplate reader. MTT assay relies on the mitochondrial metabolism for the conversion of the yellow dye MTT on a purple dye formazan by viable cells **(figure 21)**.

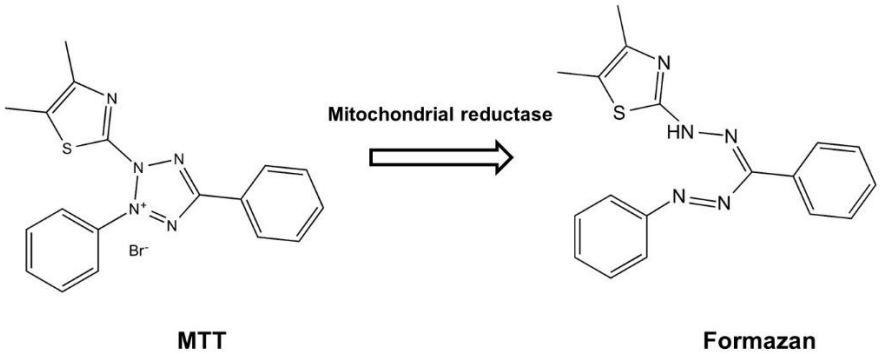

Figure 21 - Schematic illustration of MTT assay. The MTT dye is converted into the purple formazan salt by the mitochondrial reductase enzymes of viable cells.

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is only released from cells into the extracellular space when the plasma membrane is disrupted. So, the release of LDH into the culture medium was evaluated by measurement of LDH activity by the use of the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit according to the manufacturer's instructions. Released LDH activity is measured with a coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product. Released LDH catalyzes the reaction of NAD+ and lactate to pyruvate and NADH, then NADH plus INT, in the presence of diaphorase, is catalyzed to NAD+ and the red formazan product. The amount of color formed is proportional to the number of lysed cells **(figure 22).** Generation of Formazan is monitored by measuring absorbance at 490 nm. Visible wavelength absorbance data were collected using a BioTek Power Wave XS plate reader.

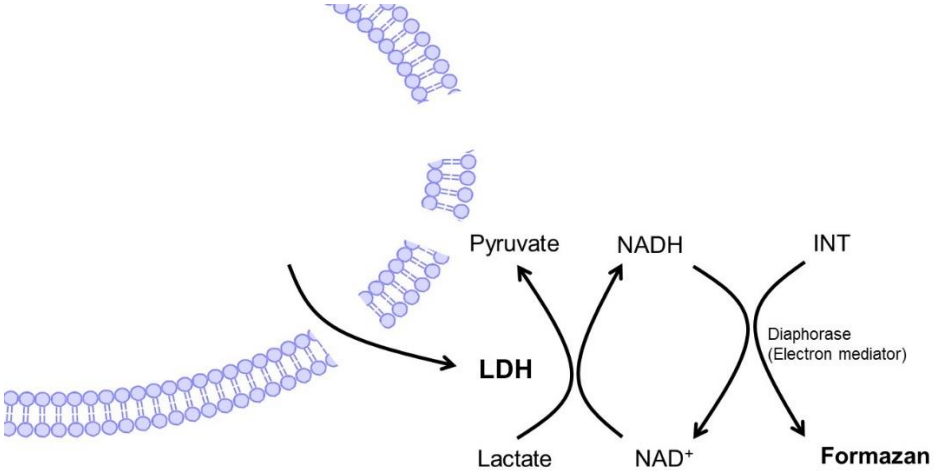

Figure 22 - Schematic illustration of LDH assay. Released LDH catalyzes the reaction of NAD+ and lactate to pyruvate and NADH, then NADH plus INT, in the presence of diaphorase, is catalyzed to NAD+ and the red formazan product.

All the experiments were performed in triplicate in at least three independent experiments and results were expressed as percentage of control/untreated cells.

4. Morphological studies

The morphological alterations caused by cannabinoids treatment were evaluated by phase-contrast microscopy, Giemsa, Höechst and Acridine Orange (AO) staining.

The cells were cultured in 24-well culture plates with coverslips and treated with the different cannabinoids (50 µM of THC, 2 µM of WIN, 10 µM of 2-AG) for 24 hours. Cells were observed under a phase contrast microscope (Eclipse 400, Nikon, Japan) equipped with an image analysis software Nikon NIS Elements.

Giemsa staining is a variant of the Romanowsky-type stain. This technique allows the evaluation of cell morphology. Giemsa contains a mixture of methylene blue, eosin and Azure B (methylene azure B). The eosin Y dye stains the basic components of the cells. The methylene blue and azure B dyes stain the acidic components in shades between blue and purple. Cells were washed with PBS and fixed with methanol for 10 minutes at 4ºC. Then cells were washed with PBS and stained with Giemsa stain solution, diluted in distilled water (1:10) for 30 minutes. After washing with tap water, the coverslips with the stained cells were dehydrated and mounted in DPX mounting medium and observed under a bright field microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software LeicaQwin.

Höechst is a dye that emits blue fluorescence when bound to DNA. This staining allows the evaluation of nuclear morphology and the identification of apoptotic nuclei, which present chromatin's condensation and fragmentation. After fixation with 4% paraformaldehyde solution for 15 minutes at 4ºC, the cells were exposed to 0.5 µg/mL Höechst 33342 (in PBS) for 20 minutes, washed with PBS, mounted in Fluoroshield mounting medium and observed under a fluorescence microscope equipped with an excitation filter with maximum transmission at 360/400 nm (Eclipse CI, Nikon, Japan). Images were processed by Nikon NIS Elements Image Software.

Acridine Orange (AO) is a cationic, lipophilic, fluorochrome stain capable of permeating cell and organelle membranes, though, once protonated, these dyes tend to become trapped on the low pH side of the membrane barrier leading to their accumulation in acidic organelle structures, such as phagosomes and lysosomes. At the end of the incubation time with the cannabinoids, cells were incubated with AO at 0.1 µg/mL for 15 min at 37ºC, washed three times with PBS, mounted in PBS and visualized immediately in a fluorescence microscope equipped with an excitation filter with maximum transmission at 360/400 nm (Eclipse CI, Nikon, Japan). Images were processed by Nikon NIS Elements Image Software.

5. Mitochondrial membrane potential (∆ψm)

For measure the mitochondrial membrane potential, cells were seeded in a 96-well black plate, pre-incubated for 30 minutes with the CB1 and CB2 antagonists, AM251 (1 µM) and AM630 (1 µM), respectively, and treated with the cannabinoids (25/50 µM of THC, 2 µM of WIN, 10 µM of 2-AG) for 24 hours. The cells were then washed with a PBS/sucrose 100 mM/protease inhibitor (PI) cocktail and incubated with a 100 nM dihexyloxacarbocyanine iodide ($DiOC_6$) solution for 20 minutes at 37°C in the dark. $DiOC_6$ is a fluorescent lipophilic cationic dye, which will accumulate into the mitochondrial membrane matrix space in inverse proportion to ∆ψm. A more polarized mitochondria (hyperpolarized, where the interior is more negative) will accumulate more dye, and depolarized mitochondria accumulate less dye. Then, the $DiOC_6$ solution was removed to eliminate the background fluorescence, the PBS/sucrose/PI solution was added to the cells and the fluorescence was measured by the use of a Microplate Fluorimeter (BioTek Instruments Winooski, VT, USA) (excitation: 488 nm; emission: 525 nm). For the positive control, the cells were incubated with the mitochondrial membrane-depolarizing agent CCCP (10 µM) for 15 minutes, at 37°C, before incubation with $DiOC_6$. The results are expressed as a percentage, comparing the loss of mitochondrial membrane potential of cannabinoid-treated cells with the untreated cells.

6. Reactive oxygen and nitrogen species

For the quantification of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) generated after treatment with cannabinoids, cells seeded in a black 96-well plate were incubated with the probe 2'-7'-Dichlorodihydrofluorescein (DCDHF-DA) for 1hour at room temperature. After the probe incubation time, cells were pre-treated for 30 minutes with the CB1 and CB2 antagonists, AM251 (1 µM) and AM630 (1 µM), respectively, and then the cannabinoids (50 µM of THC, 1/5 µM of WIN, 10 µM of 2-AG) were added and incubated for 1 hour. Inside the cell, the probe is hydrolyzed by esterases into DCDHF, which will be oxidized to the fluorescent molecule Dichlorofluorescein (DCF), by free ROS and RNS **(figure 23).** Fluorescence, proportional to the cellular levels of ROS/RNS, was measured using the Microplate Fluorimeter (BioTek Instruments) (excitation: 485±10 nm; emission: 530±12.5 nm). The stress inducer H_2O_2 (200 µM) was used as a positive control, by incubation for 20 minutes at room

temperature. The results are expressed as a percentage, comparing generation of ROS/RNS of cannabinoid-treated cells with the untreated cells.

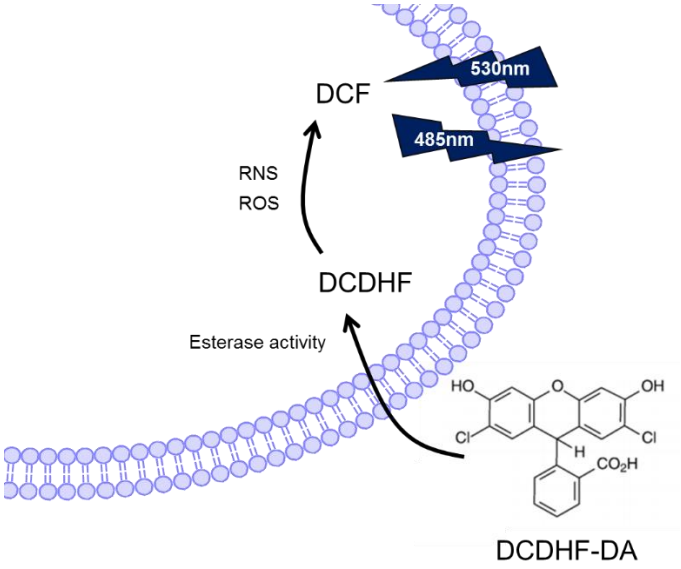

Figure 23 - Schematic illustration of the measurement of ROS/RNS generation. Inside the cell, the probe DCDHF-DA is hydrolyzed by esterases into DCDHF. Then, DCDHF is oxidized to the fluorescent molecule DCF, by free ROS and RNS.

7. Glutathione assay

Glutathione plays a critical role in the cellular defense against oxidative and nitrosative stress in mammalian cells. Glutathione exists in reduced (GSH) and oxidized (GSSG) forms. Reduced glutathione (GSH) is a major tissue antioxidant that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water. In the GPx catalyzed reaction, the formation of a disulfide bond between two GSH molecules gives rise to oxidized glutathione (GSSG). The enzyme glutathione reductase (GR) recycles GSSG to GSH with the simultaneous oxidation of ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH2). To measure the level of total glutathione (GSSG + GSH) of the sample it was used a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB. The oxidized glutathione formed is recycled by glutathione reductase and NADPH. The product, TNB, is assayed colorimetrically at 412 nm. This reaction is used to measure the

reduction of GSSG to GSH and the rate of the reaction is proportional to the GSH and GSSG concentration. To measure the levels of GSSH, GSH was masked by pretreatment with 2-vinylpyridine (2-VP) **(figure 24).**

For this assay, BeWo cells were seed in a 6-well plate and treated with the cannabinoid 2-AG for 24 hours. At the end of incubation time, cells were first deproteinized with an $HClO_4$ 5% acid solution, scrapped and collected to an Eppendorf. After centrifugation (6000 g, 5 minutes, 4ºC), both glutathione forms were measured in the supernatant and the total amount of protein was quantified, after addition of NaOH 0,5 M to the pellet, by Lowry method. The results were expressed by the total protein content.

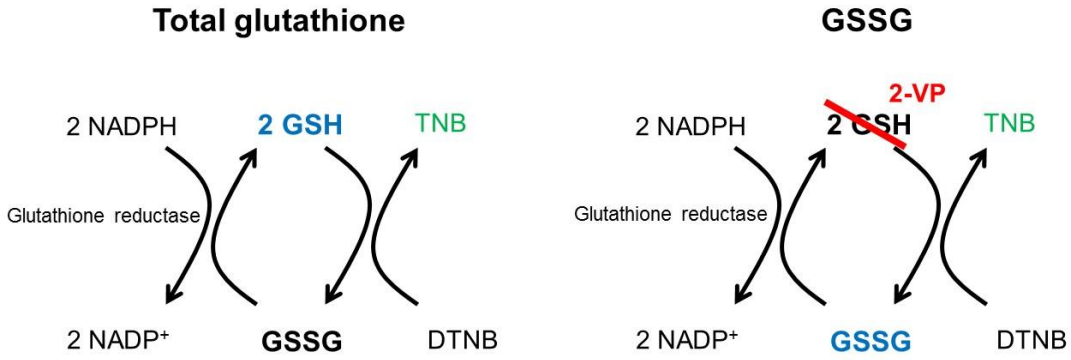

Figure 24 - Schematic illustration of glutathione assay. Total glutathione of the sample was measured used a kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of DTNB to TNB. The oxidized glutathione formed is recycled by glutathione reductase and NADPH. The product, TNB, is assayed colorimetrically at 412 nm. To measure the levels of GSSH, GSH was masked 2-VP.

8. Western Blot

To detect changes in CHOP expression levels after cannabinoid treatment, it was performed a western blot technique.

BeWo cells and CTs were seeded in 6-well plates, pre-incubated for 30 minutes with the CB1 and CB2 antagonists, AM251 (1 µM) and AM630 (1 µM), respectively, and treated with the compounds for 24 hours. After the incubation period, RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) plus a cocktail of protease inhibitor (1:100) was added to each well, and incubated on ice for 10 minutes. Then, the cells were scrapped, collected to an Eppendorf, subjected to three cycles of freezing/thawing and centrifuged

at 14000 g for 10 minutes at 4°C. Total protein concentrations were measured by Bradford assay.

Protein samples (30 µg) were prepared in sample buffer, denatured by boiling for 3 minutes, subjected to SDS–polyacrylamide (10%) and transferred onto nitrocellulose membranes for 45 minutes in a semi dry transfer system (Trans-Blot SD semi dry transfer cell, Bio-Rad). After blocking non-specific binding sites with blocking buffer (5% of dry milk in PBS with Triton X-100, 0.1%), the membranes were incubated with the primary antibody anti-CHOP (1:100) overnight at 4°C. Then, after washed with PBS 0.1% Triton, the membranes were incubated for 1 hour, at room temperature, with peroxidase-conjugated secondary antibody anti-rabbit (1:1000). Finally, the blots were analysed using a chemiluminescence detection kit and exposed to X-ray films. The membranes were then stripped and reincubated with anti-β-tubulin (1:500) for a loading control.

9. Caspase 3/7 activity

To detect caspase 3/7 activity, the cells were seeded in a 96-well white plate pre-incubated for 30 minutes with the CB1 and CB2 antagonists, AM251 (1 µM) and AM630 (1 µM), respectively, and treated with the cannabinoids for 24 hours. At the end of the incubation time, Caspase-Glo 3/7 reagent was added to the cells according to the manufacturer's instructions. The plate was incubated at room temperature for 1 hour and the resultant luminescence was measured in relative light luminescence units (RLU) using the 96-well Microplate Luminometer (BioTek Instruments, Winooski, VT, USA) **(figure 25).** A negative control assay was conducted by co-incubation with a pan-caspase inhibitor, Z-VAD-FMK (10 µM), and a positive control assay was conducted using Staurosporine (0.1 µM), which was added 12 hours before the end of the experiment. The results are expressed as a percentage, comparing caspase 3/7 activity of cannabinoid-treated cells with the untreated cells.

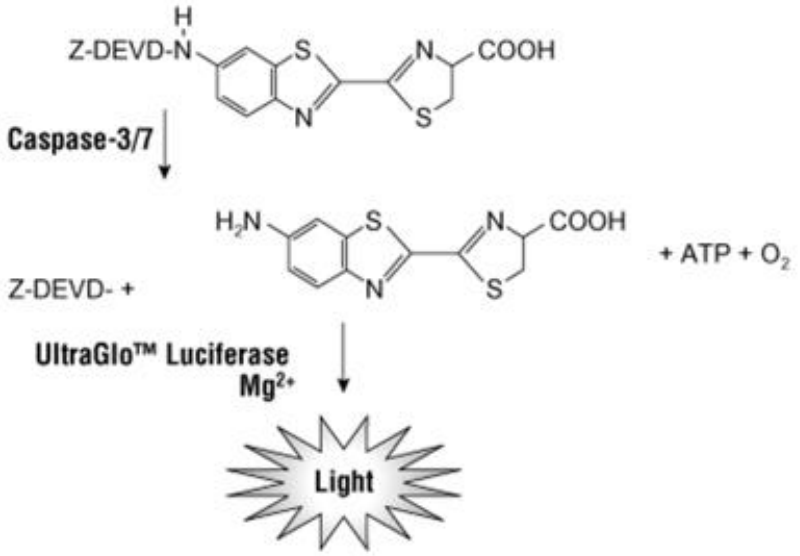

Figure 25 - Schematic illustration of caspase 3/7 detection assay.

10. Cell cycle analysis

BeWo cells were seeded in 6-well plates and treated as described. After 24 hours of treatment cells were tripsinized, washed with PBS, resuspended in 0.5 mL of PBS and fixed with 4.5 mL of cold ethanol 70%, on ice, for at least 24 hours. After this step, cells were centrifuged at 600 g and washed with PBS to remove the fixative solution. Then, 400 µL of a DNA staining solution [5 µg/mL Propidium Iodide (PI), 0.1% Triton X-100 and 200 µg/mL Dnase-free Rnase A in PBS] was added to the pellet and the cells were incubated overnight at 4ºC and protected from light. After this, samples were filtered and DNA content was analysed by flow cytometry based on the acquisition of 40 000 events (with a threshold of 100 000) in a BD Accuri™ C6 (Becton-Dickinson, San Jose, CA, USA) equipped with BD Accuri C6 software. Detectors for the three fluorescent channels (FL-1, FL-2, FL-3) and forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris, cell doublets and aggregates were gated out and the singlet cells were analysed using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. The antiproliferative effect was indicated by the percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle. Data was analysed using BD Accuri C6 software. Assays were performed in triplicate in three independent experiments. Results are expressed as percentage of total cells in each cell cycle phase.

11. Statistical analysis

Statistical analysis was carried out by ANOVA, followed by the Tukey post hoc test to make pairwise comparisons of individual means when significance was indicated (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at least three independent experiments carried out in triplicate. Data are expressed as the mean±SEM, and differences were considered to be statistically significant at $P<0.05$.

Chapter III

Results

1. Cannabinoid effects in BeWo cells

1.1 Cell viability assays

In order to investigate the impact of cannabinoids in BeWo cells viability, it was compared the effects of the CB agonists THC, WIN and 2-AG.

In previous works from the group, it was observed that 2-AG decreased cell viability in a dose-dependent manner [129]. In that way, it was investigated THC and WIN effects by using different concentrations (1-50 µM) for 24 hours.

To analyze the effects on cell viability, it was performed the MTT assay and measured the activity of the enzyme LDH released in cell culture medium.

The phytocannabinoid **THC** did not seem to cause changes in cell viability **(figure 26)**.

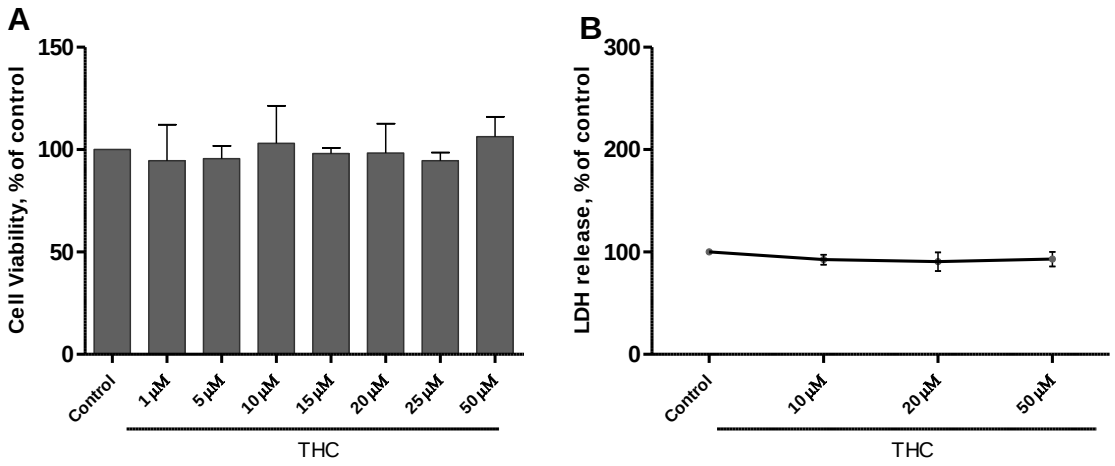

Figure 26 - Effect of THC on BeWo cells viability. Cell viability of THC-treated cells with different concentrations (10-50 µM) at 24 hours of treatment, assessed by **(A)** MTT assay and **(B)** LDH release. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate.

WIN induced a decrease in cell viability dependent on the concentration of the compound. The MTT results showed that 2 µM of WIN induced a decrease of 16% in cell viability, though no significant release of the cytoplasmic LDH was observed **(figure 27**).

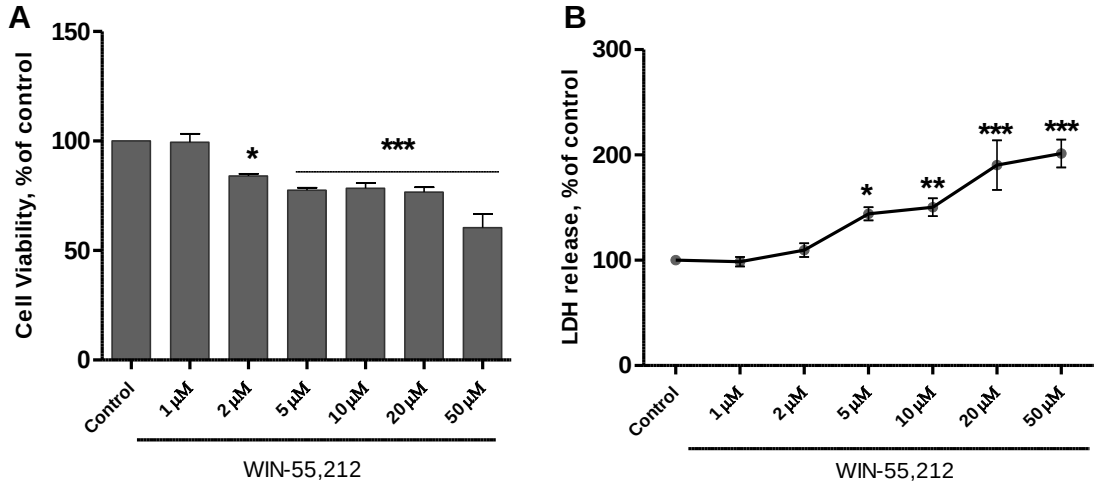

Figure 27 - Effect of WIN on BeWo cells viability. Cell viability of WIN-treated cells with different concentrations (1 - 50µM) at 24h of treatment, assessed by **(A)** MTT assay and **(B)** LDH release. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *($p<0.05$), ** ($p<0.005$) and *** ($p<0.0005$).

1.2 Morphological studies

To investigate whether the different cannabinoids induced morphological alterations, BeWo cells were treated for 24 hours with each compound, observed under a phase contrast microscope and stained with Giemsa and Höechst.

Treatment with 50 µM of **THC** did not cause alterations in cell morphology **(figure 28).** In A, the typical appearance of control (untreated) BeWo cells is presented.

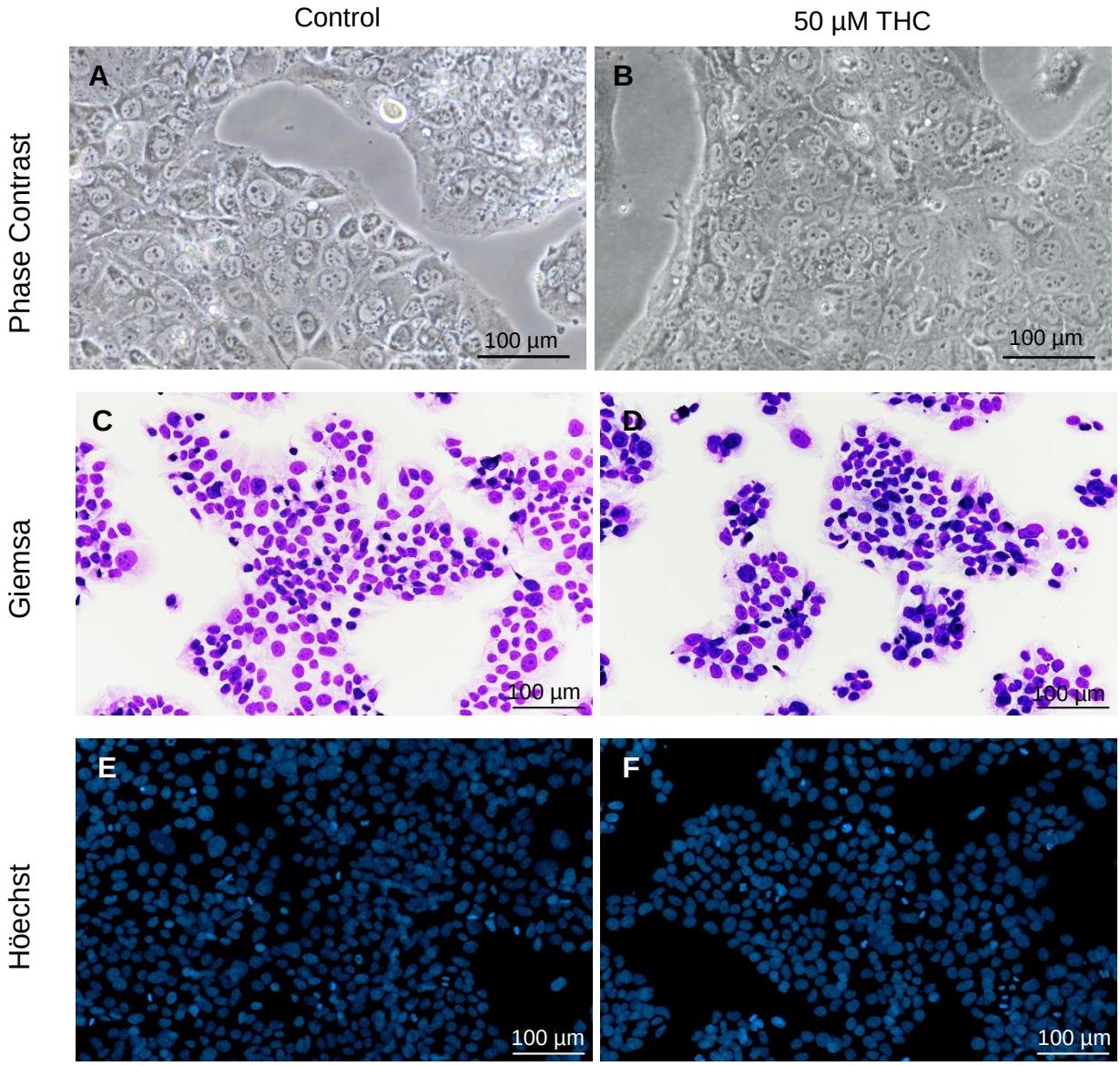

Figure 28 - Effects of THC on BeWo cells morphology. (A, B) Phase-contrast microscopy; **(C, D)** Giemsa staining; **(E, F)** Höechst staining. BeWo cells morphology was analyzed in the absence (control) or presence of THC (50 µM) after 24 hours. Results are shown from single representative of three independent experiments.

On the other hand, cells exposed to **WIN** (2 µM) exhibited morphological alterations **(figure 29).** In phase contrast microscopy it was possible to observe cytosolic vacuolization, whereas chromatin condensation and cytoplasmic vacuoles were observed by Giemsa staining. Höechst staining confirmed the presence of chromatin condensation. These alterations were accompanied by a reduction in cell density.

As WIN-treatment induced cytosolic vacuolization, a morphological analysis was undertaken in order to clarify their nature. To monitor the formation of acidic vesicular organelles (AVOs), BeWo cells were stained with Acridine Orange, a cell-permeable acidotropic dye that once protonated gets trapped inside low pH vesicles such as autophagosomes and lysosomes, emitting yellow/orange/red fluorescence. Cells were observed under a fluorescence microscope and WIN-treated cells presented alterations in green fluorescence to orange/red fluoresce, confirming the presence of AVOs **(figure 29 G, H)**, which suggests the occurrence of autophagy. Nevertheless, further studies must be performed to quantify the increase in the formation of AVOs, as well as the expression of biochemical markers.

As already referred, the endocannabinoid **2-AG** was previously analyzed for cell viability and, in accordance to those previous studies, a concentration of 10 µM was selected. It was possible to observe the presence of chromatin condensation by Giemsa and Höechst staining **(figure 30).**

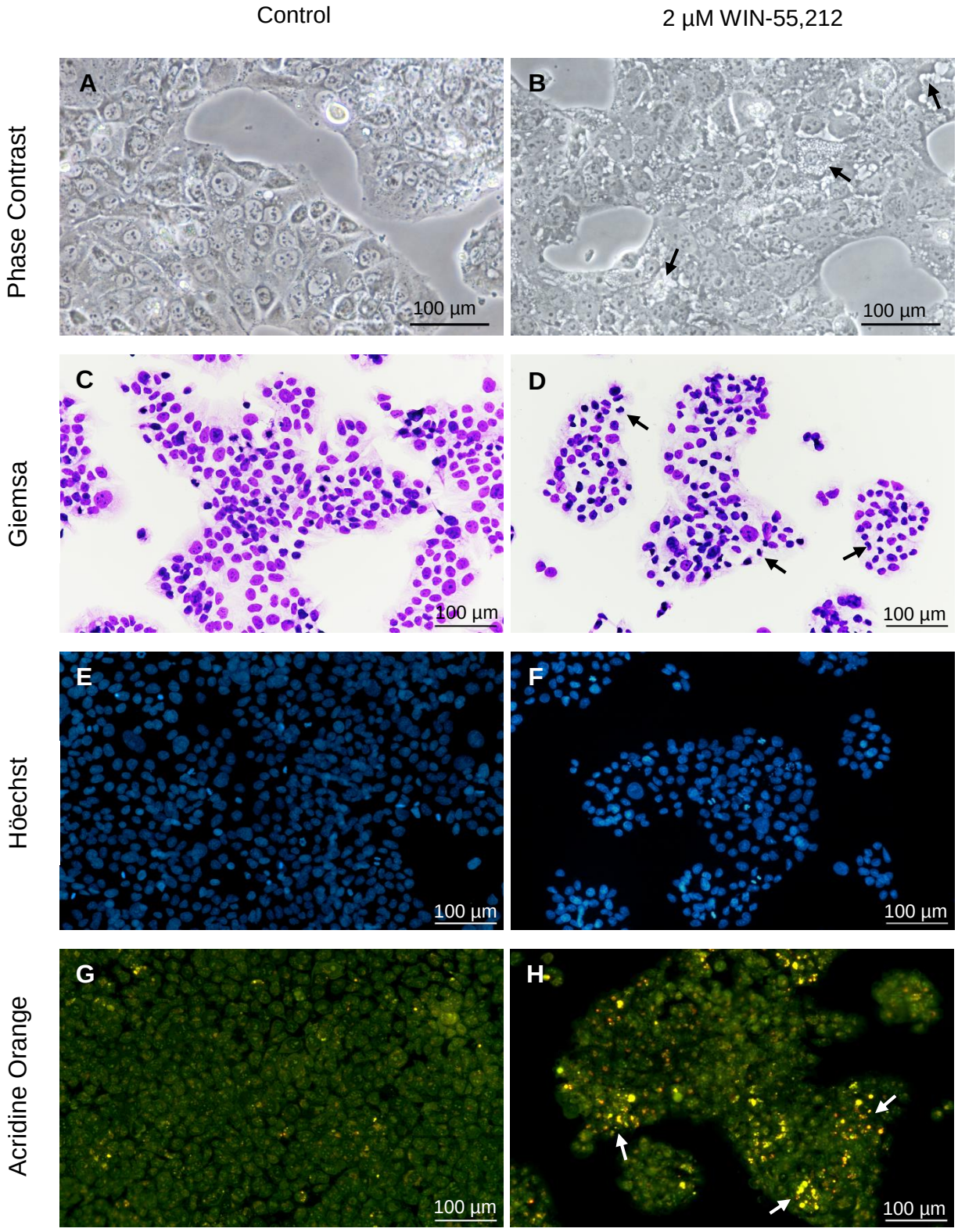

Figure 29 - Effects of WIN on BeWo cells morphology. (A, B) Phase-contrast microscopy; **(C, D)** Giemsa staining; **(E, F)** Höechst staining; **(G, H)** Acridine Orange staining. BeWo cells morphology was analyzed in the absence (control) or presence of WIN (2 µM) after 24 hours. Arrows represent vacuolization (B), chromatin condensation (D) and acid vesicles (H). Results are shown from single representative of three independent experiments.

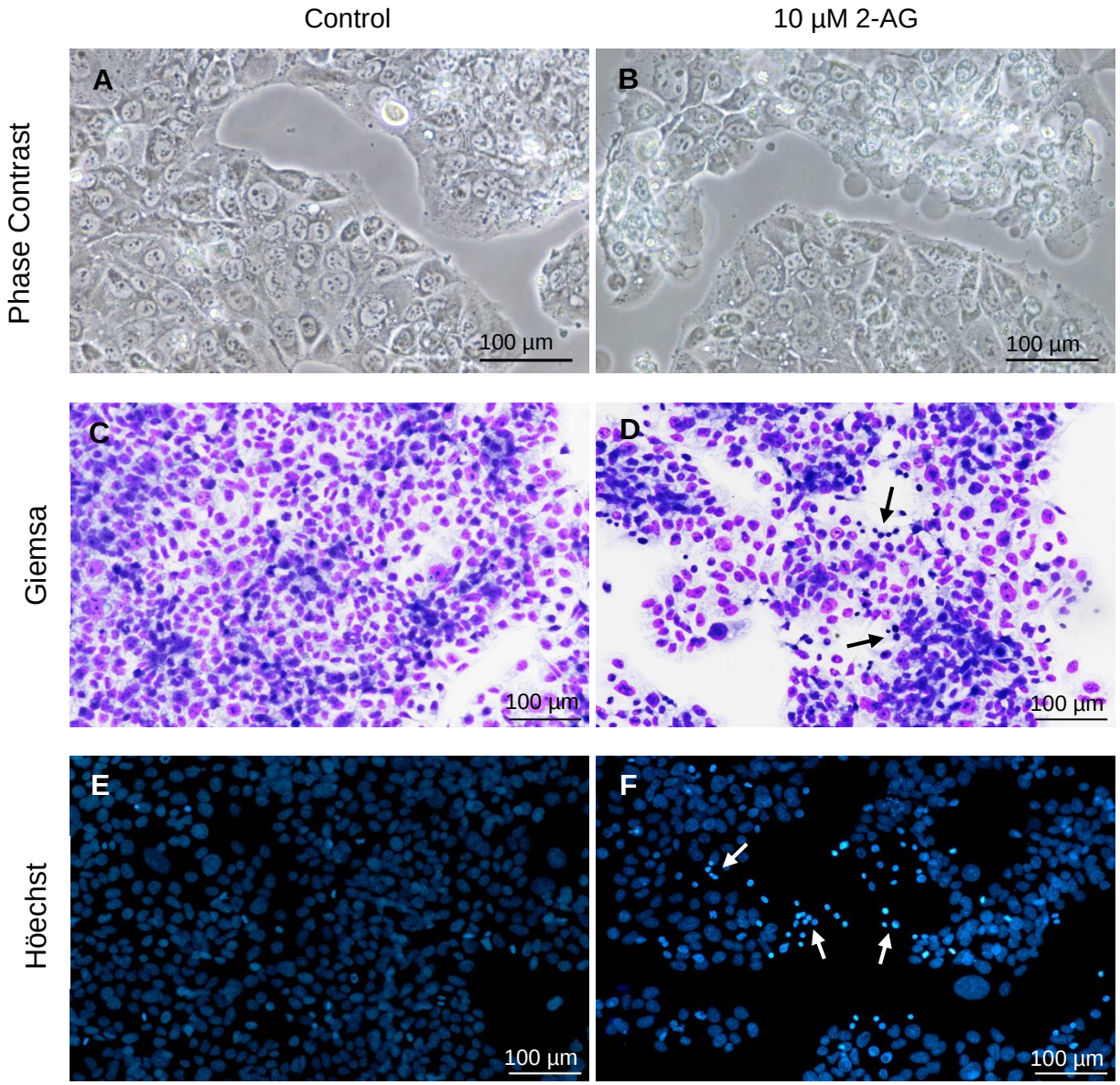

Figure 30 - Effects of 2-AG on BeWo cells morphology. (A, B) Phase-contrast microscopy; **(C, D)** Giemsa staining; **(E, F)** Höechst staining. BeWo cells morphology was analyzed in the absence (control) or presence of 2-AG (10 µM) after 24hours. Arrows represent chromatin condensation (D, F). Results are shown from single representative of three independent experiments.

1.3 Mitochondrial membrane potential

The capacity of the cannabinoids to induce alterations in the mitochondrial membrane potential (Δψm) was assessed by fluorimetry with the probe $DiOC_6$. All the cannabinoids induced similar mitochondrial membrane potential loss. In the case of THC, concentrations of 25 µM or 50 µM induced a decrease in Δψm around 38%, for 2 µM WIN a decrease of 33% and for 10 µM 2-AG of 38% **(figure 31).**

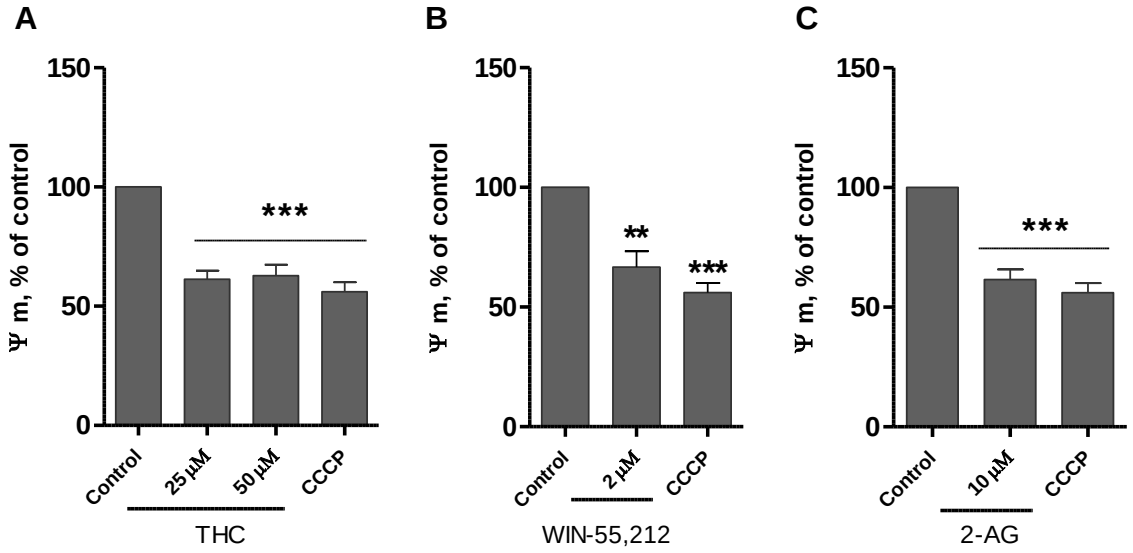

Figure 31 - Effect of THC, WIN and 2-AG on BeWo cells mitochondrial membrane potential at 24h. **(A)** Changes in mitochondrial membrane potential induced by THC treatment at 25 µM and 50 µM; **(B)** WIN treatment at 2 µM; **(C)** 2-AG treatment at 10µM. CCCP (10µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as ** ($p<0.005$) and *** ($p<0.0005$).

Such results suggest that the effects of WIN and 2-AG on cell viability can be associated with mitochondrial disfunction. To understand the involvement of CB receptors in the observed changes of mitochondrial membrane potential, WIN or 2-AG were treated with the CB1 antagonist AM251 (1 µM) and the CB2 antagonist AM630 (1 µM). Since THC did not induced changes in cell viability, in this case the studies with the CB antagonists were nor performed.

CB antagonists did not cause significant reversion of the WIN-induced reduction in Δψm. AM251 caused a reversion of 2.3% and AM630 of 6.3% **(figure 32).**

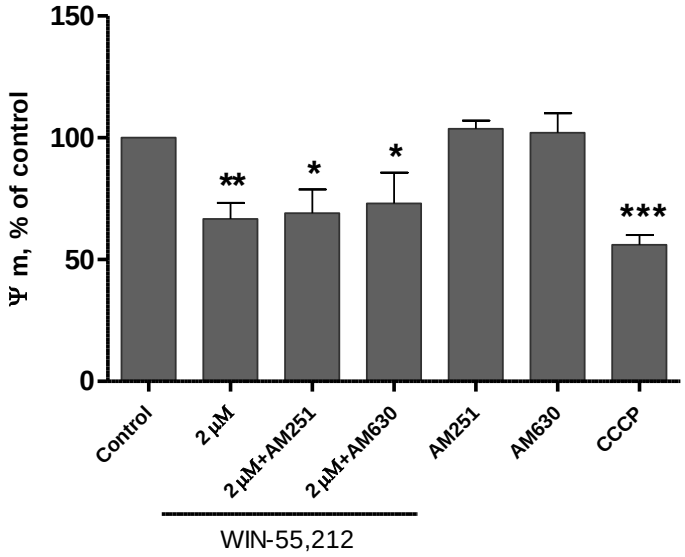

Figure 32 - Effect of 2 µM WIN on BeWo cells mitochondrial membrane potential at 24 hours in combination with CB antagonists. Effect of the addition of CB antagonists AM251 and AM630 at 1 µM. CCCP (10 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *(p<0.05), ** (p<0.005) and *** (p<0.0005).

Like in the case of WIN treatments, 2-AG in combination with the CB antagonists showed no reversion of the effects caused by 2-AG alone **(figure 33).**

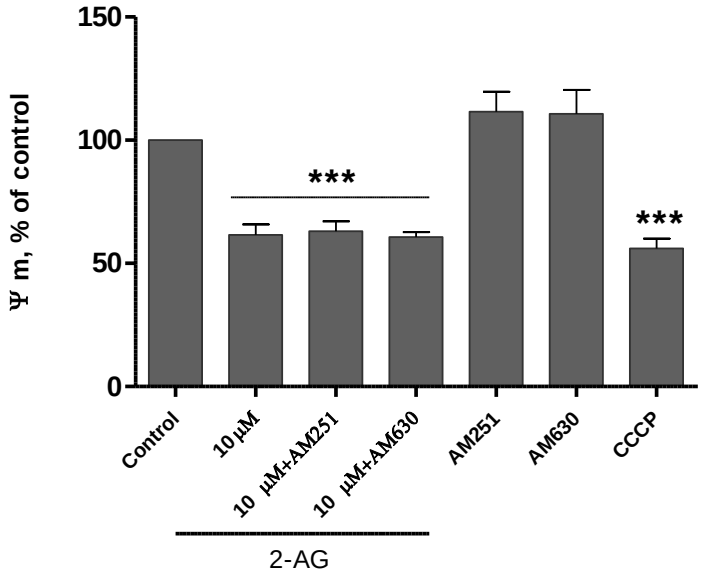

Figure 33 - Effect of 10 µM of 2-AG on BeWo cells mitochondrial membrane potential at 24 hours in combination with CB antagonists. Effect of the addition of CB antagonists AM251 and AM630 at 1 µM. CCCP (10 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** (p<0.0005).

1.4 Reactive oxygen and nitrogen species

To further explore the underlying mechanisms of cell viability loss, it was evaluated reactive species production, which is often associated with mitochondrial dysfunction and oxidative stress. The ability of the cannabinoids to induce the production of ROS/RNS was evaluated by fluorimetry with the probe DCDHF-DA.

The endocannabinoid 2-AG was the only compound that induced the production of ROS/RNS **(figure 34 C).** The treatment with 10 µM 2-AG induced a 69% increase in ROS/RNS generation.

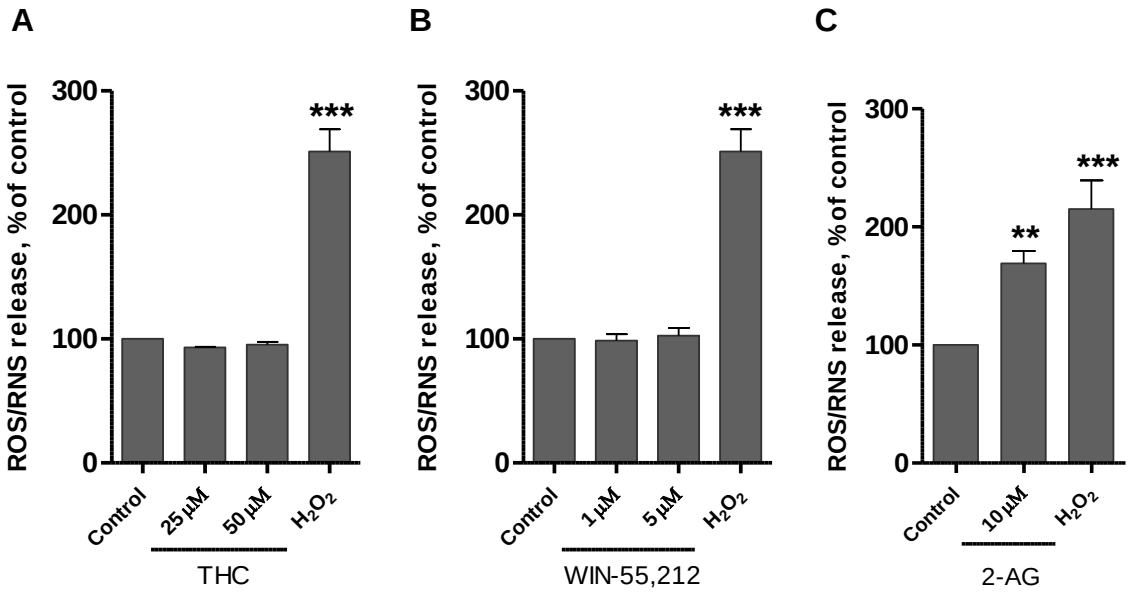

Figure 34 - Effect of THC, WIN and 2-AG on BeWo cells ROS/RNS generation. (A) Production of ROS/RNS induced by THC at 25 µM and 50 µM; **(B)** by WIN at 1 µM and 5 µM; **(C)** by 2-AG at 10 µM. H_2O_2 (200 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as ** ($p<0.005$) and *** ($p<0.0005$).

The addition of AM251 and of AM630 did not revert significantly the 2-AG-induced effect **(figure 35).**

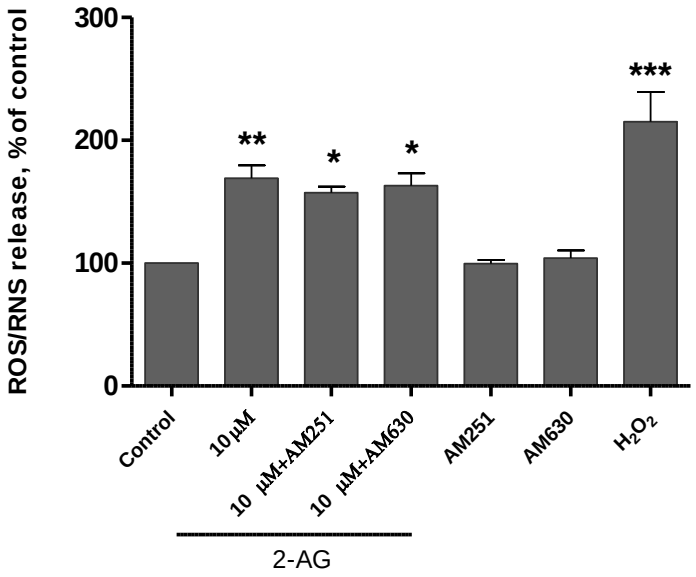

Figure 35 - Effect of 2-AG in combination with the CB antagonists on BeWo cells ROS/RNS generation. Production of ROS/RNS induced by 2-AG treatment at 10 µM in the presence of CB antagonists AM251 and AM630 at 1 µM. H_2O_2 (200 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *($p<0.05$), ** ($p<0.005$) and *** ($p<0.0005$).

1.5 Glutathione levels

Since glutathione plays a critical role in the cellular defense against oxidative and nitrosative stress in mammalian cells and 2-AG induced a dramatic increase in ROS/RNS production, the ratio between the oxidized and reduced form of glutathione was measured after 2-AG treatment. In healthy cells, more than 90% of the total glutathione pool is in the reduced form (GSH). When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSSG to GSH increases. Thus, an increased ratio of GSSG-to-GSH is an indication of oxidative stress. The monitoring of reduced and oxidized GSH in biological samples is essential for evaluating the redox and detoxification status of the cells and tissues against oxidative and free radicals mediated cell injury.

The preliminary results suggest that the treatment with 10 µM of 2-AG did not seem to cause changes in the ratio GSSH/GSH **(figure 36).**

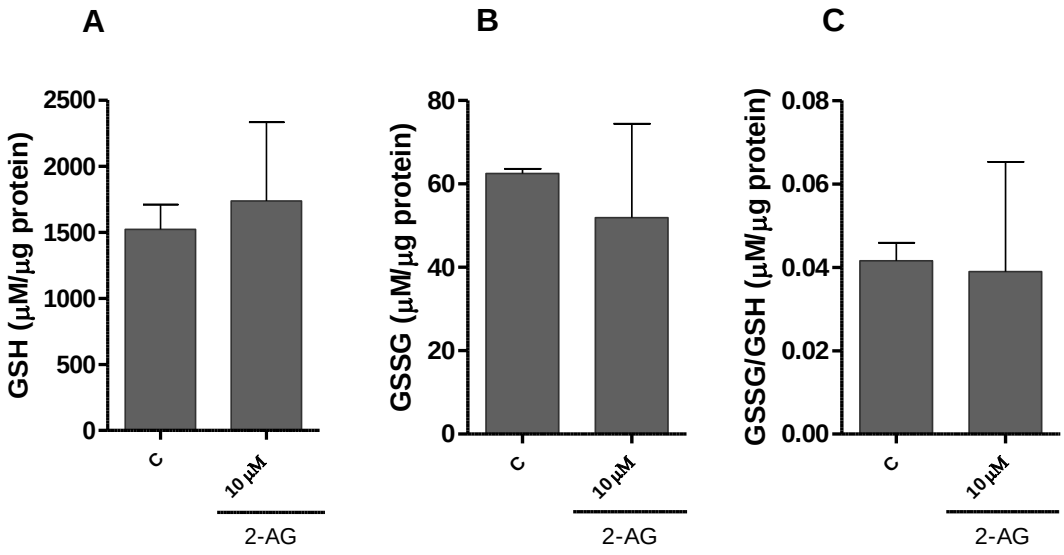

Figure 36 - Preliminary results of the effect of 2-AG (10 µM) on BeWo cells glutathione levels at 24 hours. (A) Total glutathione levels (GSH); **(B)** oxidised form of glutathione (GSSG); **(C)** GSH/GSSG ratio.

1.6 CHOP expression

Since 2-AG induces oxidative stress in BeWo cells, we analysed CCAAT-enhancer-binding protein homologous protein (CHOP) expression by western blot. CHOP is a protein that is induced by ER-stress and causes downregulation of the anti-apoptotic protein Bcl-2, favouring a pro-apoptotic drive at the mitochondria by proteins that cause mitochondrial damage, cytochrome *c* release and caspase-3 activation.

Preliminary results showed an increase in CHOP expression after 10 µM 2-AG treatment, an effect that appears to be reverted by the co-incubation with the CB2 antagonist AM630 **(figure 37).**

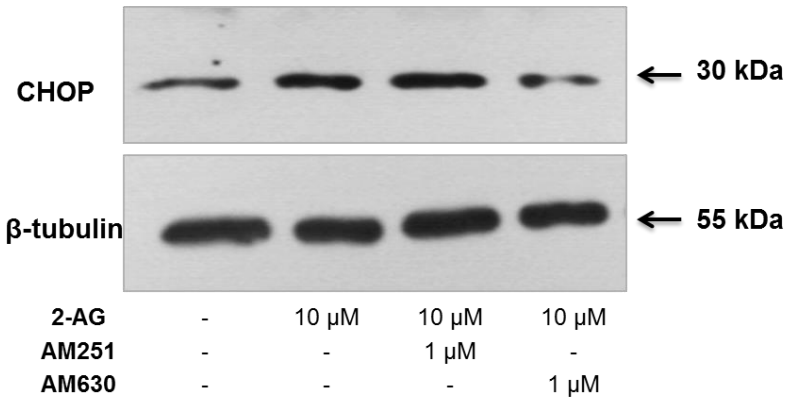

Figure 37 - CHOP expression in BeWo cells. Western Blot analysis of CHOP expression in the absence (control) or presence of 2-AG (10 µM), and 2-AG (10 µM) co-incubated with the CB antagonists AM251 (1 µM) and AM630 (1 µM). β-tubulin was used as loading control.

1.7 Cell cycle analysis

A possible mechanism responsible for the observed effects of WIN and 2-AG in cell viability reduction is the deregulation of the cell cycle and subsequent alterations in cell proliferation. In that way, cell cycle progression was analyzed by flow cytometry after PI staining. According to DNA content, cells were distributed to the correspondent cell cycle phases. After 24 hours of treatment, 2 µM WIN induced a significant cell cycle arrest in the G_0/G_1 phase (64%), when compared to control cells (51%). Such increase was accompanied by a decrease in S phase (10% *vs* 16% for control) and in G_2/M phase (27% *vs* 32%) **(figure 38 and 39).**

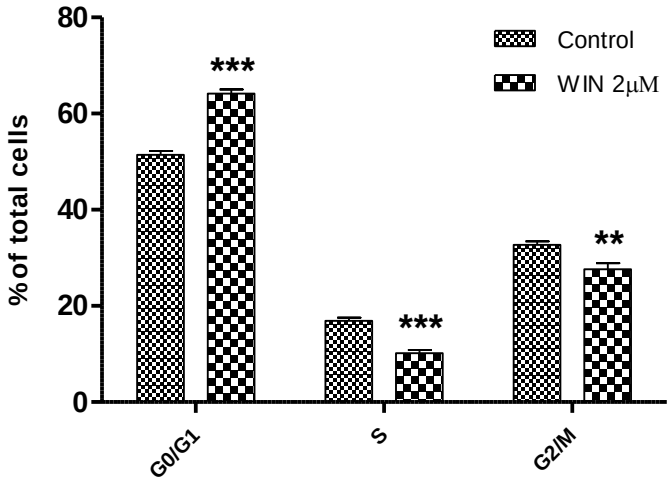

Figure 38 - Effects of 2 µM of WIN on BeWo cell cycle progression after 24 hours of treatment. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as ** ($p<0.005$) and *** ($p<0.0005$).

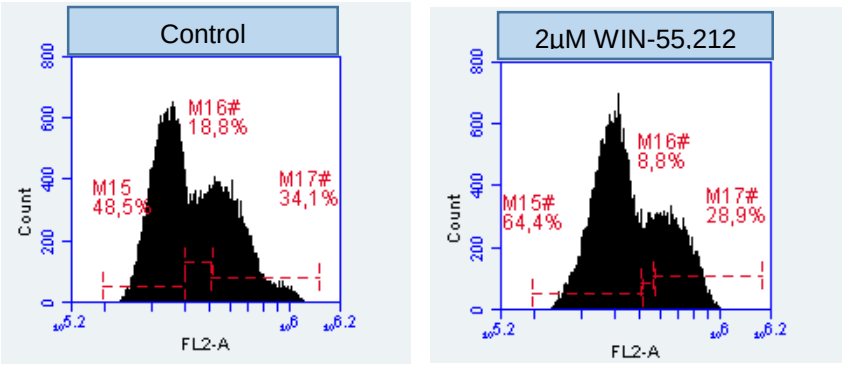

Figure 39 - Representative histograms of cell cycle distribution of BeWo cells control and treated with 2 µM of WIN for 24 hours. Histograms were obtained with BD Accuri C6 software and are representative of one independent assay. The numbers indicate the percentage of cells in each cell cycle phase: M15 is the G_0/G_1 phase, M16 represents the S phase and M17 the G_2/M.

On the other hand, 10 µM of 2-AG induced a significant cell cycle arrest in the G_2/M phase (37%), when compared to control cells (32%). This retention may be associated with apoptosis. Such increase was compensated by a decrease in S phase (11% *vs* 16% on control) **(figure 40 and 41).**

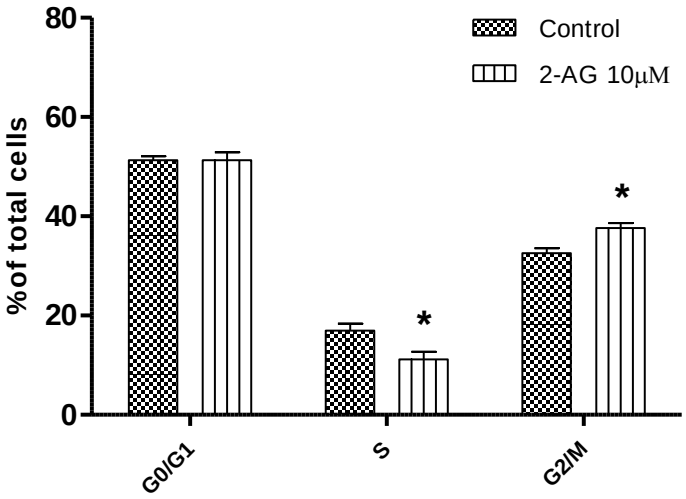

Figure 40 - Effects of 10 µM of 2-AG on BeWo cell cycle progression after 24 hours of treatment. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *(p<0.05).

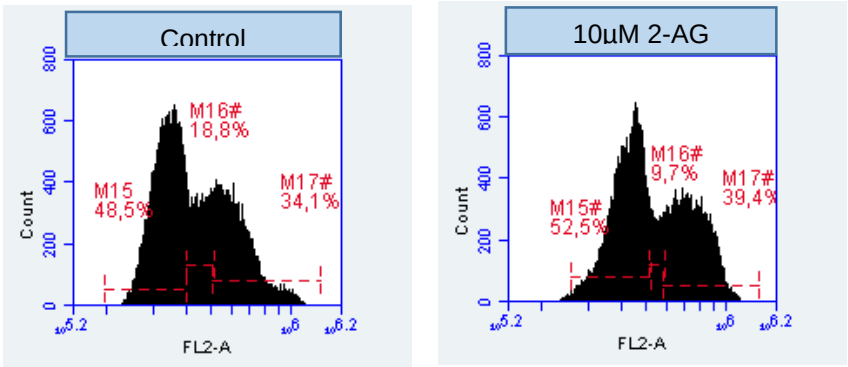

Figure 41 - Representative histograms of cell cycle distribution of BeWo cells control and treated with 10 µM of 2-AG for 24 hours. Histograms were obtained with BD Accuri C6 software and are representative of one independent assay. The numbers indicate the percentage of cells in each cell cycle phase: M15 is the G_0/G_1 phase, M16 represents the S phase and M17 the G_2/M.

2. Cannabinoid effects in primary cultures of human cytotrophoblasts

The effects of THC, WIN and 2-AG were also evaluated in primary cultures of cytotrophoblasts, obtained from human term placentas.

2.1 Mitochondrial membrane potential (∆ψm)

After 24h of treatment, all the compounds induced mitochondrial membrane potential loss**.** Preliminary results showed that THC treatment induced a decrease around 47% in ∆ψm **(figure 42 A)** and WIN induced a decrease around 44% **(figure 42 B),** in comparison with the control cells. 2-AG treatment induced a significant decrease of 20% in ∆ψm, compared with control **(figure 42 C).**

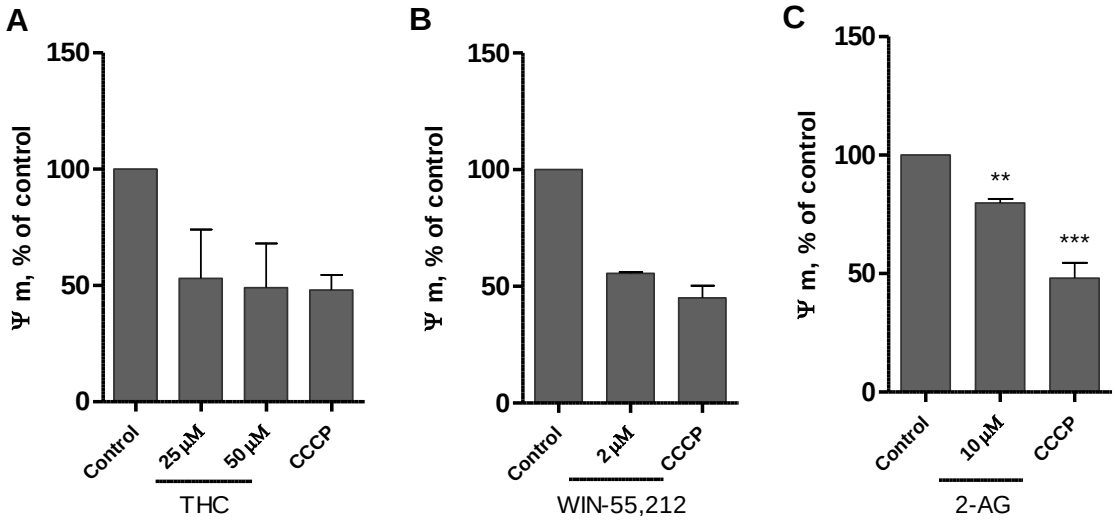

Figure 42 - Effect of THC, WIN and 2-AG on primary cultures of human cytotrophoblasts mitochondrial membrane potential at 24 hours. Preliminary results of the changes in mitochondrial membrane potential induced by **(A)** THC treatment (25 µM and 50 µM); **(B)** WIN treatment (2 µM); **(C)** 2-AG treatment (10 µM) CCCP (10 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as **($p<0.005$), and *** ($p<0.0005$).

Although preliminary data, this results suggest that, in CT, THC and WIN treatment lead to similar consequences to the ones observed in BeWo cells. Since 2-AG proved to induce apoptosis in BeWo cells, its effects were explored in CTs.

In combination with 2-AG, AM251 restored the Δψm in 11% and AM630 induced a significant reversion of 15% **(figure 43).**

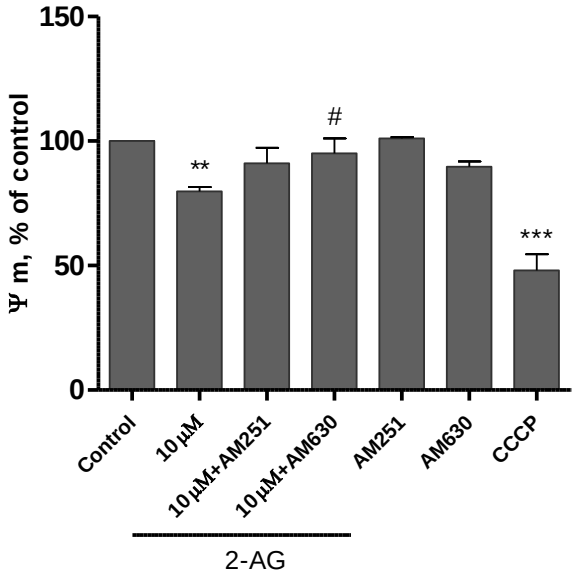

Figure 43 - Effect of 2-AG in combination with CB antagonists on primary cultures of human cytotrophoblast cells mitochondrial membrane potential at 24 hours. Changes in mitochondrial membrane potential induced by 2-AG treatment (10μM) and in combination with the CB antagonists AM251 and AM630 (1μM). CCCP (10μM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as ** ($p<0.005$) and *** ($p<0.0005$). Significant differences between 10μM 2-AG-treated cells and 2-AG (10μM) in combination with AM630 are denoted as #($p<0.05$).

2.2 Reactive oxygen and nitrogen species

2-AG was the only cannabinoid that showed to induce in BeWo cells an increase in reactive species generation. In the case of CTs, 2-AG induced an increase of 46% in ROS/RNS generation, compared with control cells, though no reversion was observed when the cells were co-treated with the CB antagonists AM251 and AM630 **(figure 44)**.

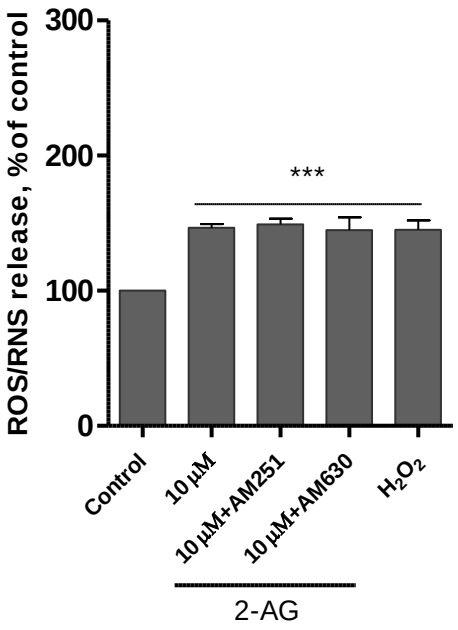

Figure 44 - Effect of 2-AG on primary cultures of human cytotrophoblasts ROS/RNS generation. Production of ROS/RNS induced by 2-AG treatment (10 µM) and in co-incubation with the CB antagonists AM251 and AM630 (1 µM). H_2O_2 (200 µM) was used as a positive control. Results are expressed as mean ± SEM of at least three independent experiments performed in triplicate. Significant differences between control and treated cells are denoted as *** ($p<0.0005$).

2.3 Morphological studies

The morphology of hCT was also analyzed, to understand if the features of apoptosis were present in these cells. Giemsa and Höechst staining showed condensation of chromatin after 24 hours of 2-AG treatment **(figure 45).** The results obtained in CT cells were in accordance with the results in BeWo cells.

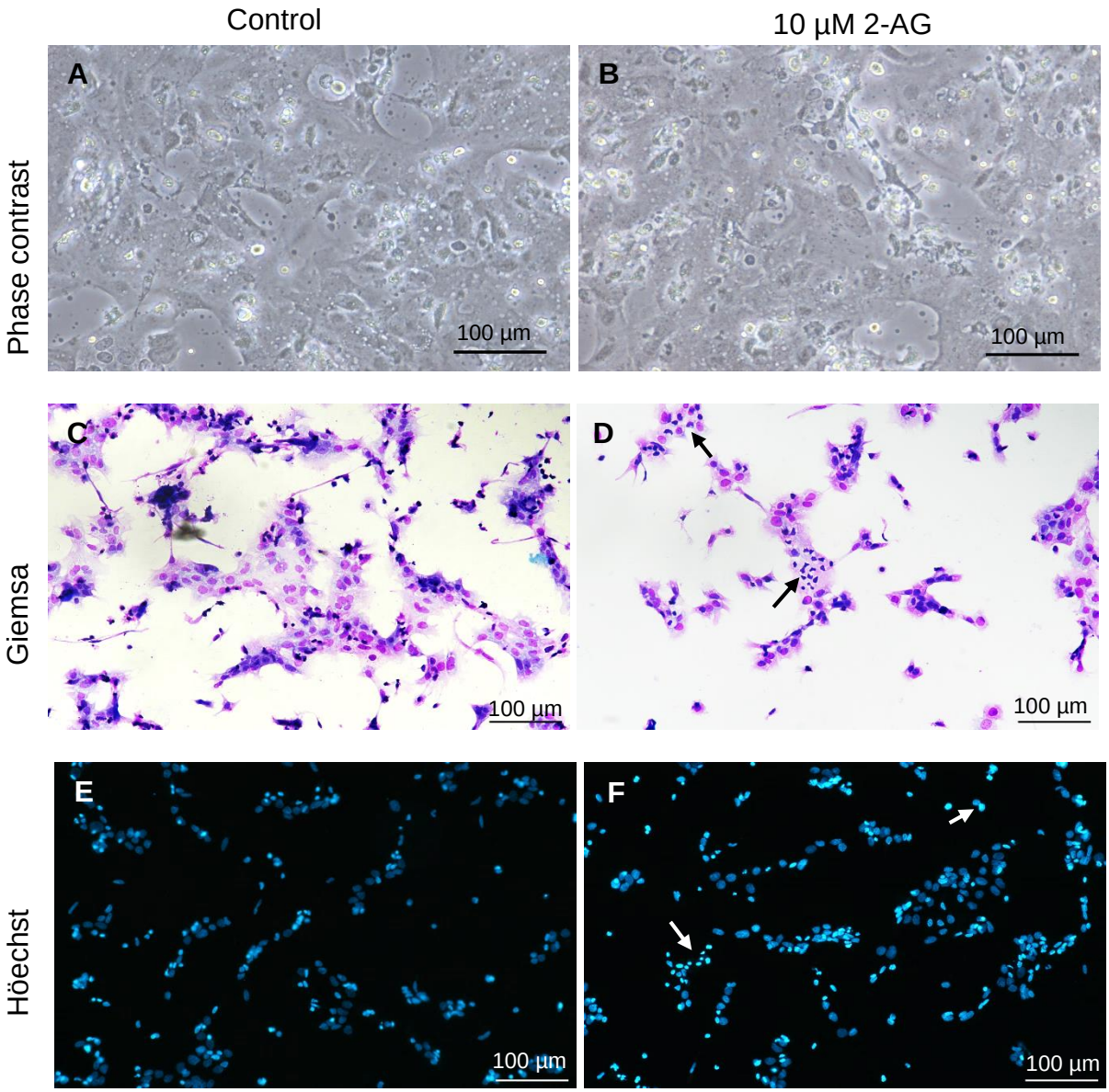

Figure 45 - Effects of 2-AG on primary cultures of human cytotrophoblasts morphology. (A, B) Phase-contrast microscopy; **(C, D)** Giemsa staining; **(E, F)** Höechst staining. BeWo cells morphology was analyzed in the absence (control) or presence of 2-AG (10 µM) after 24 hours. Arrows represent chromatin condensation (D, F). Results are shown from single representative of three independent experiments.

2.4 CHOP expression

Since CHOP expression seemed to be enhanced in BeWo cells after 24 hours of 2-AG (10µM) treatment, we investigated if the same occurred in CTs. Again, a preliminary result an increase in the expression of this ER-stress-related protein. The effect was also reversed by co-incubation with the CB antagonists, although the reversion appears to be more significant when cells were co-incubated with AM630 **(figure 46).**

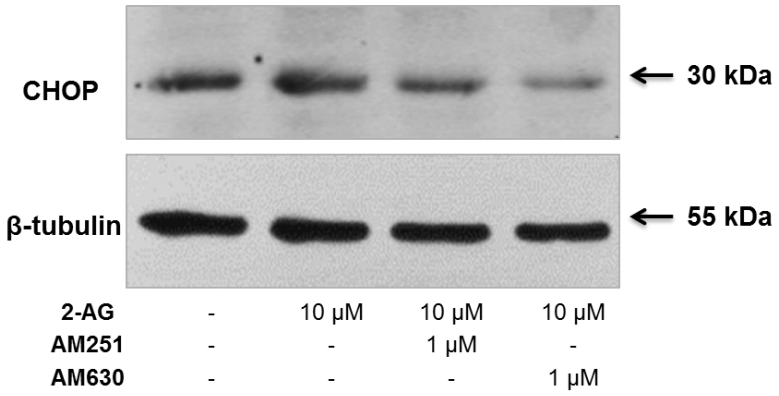

Figure 46 - CHOP expression in primary cultures of human cytotrophoblasts. Western Blot analysis of CHOP expression in the absence (control) or presence of 2-AG (10 µM), and 2-AG (10 µM) co-incubated with the CB antagonists AM251 (1 µM) and AM630 (1 µM). β-tubulin used as loading control.

2.5 Caspase 3/7 activity

In order to clarify the involvement of apoptosis in 2-AG-mediated effects, caspase 3/7 activity was measured. A preliminary result showed an increase in caspases activity after 24 hours of 2-AG treatment. At 10 µM, 2-AG induced an increase of around 33% on caspase 3/7 activity, an effect that was partially reversed by co-incubation with AM251 and AM630, which resulted in 7% and 12% of reversion **(figure 47).**

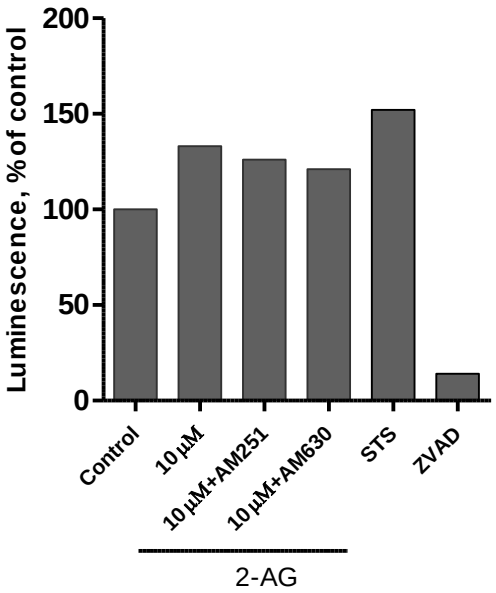

Figure 47 - Effect of 2-AG on primary cultures of human cytotrophoblasts caspase 3/7 activity at 24 hours. Changes in caspase 3/7 activity induced by 24 hours of 2-AG treatment at 10 µM and in combination with the CB antagonists AM251 and AM630 at 1 µM. ZVAD (10 µM) was used as a negative control and STS (100 nM) was used as a positive control.

Chapter IV

Discussion

During placental development, trophoblast cells undergo highly regulated processes of proliferation, apoptosis and differentiation. Proliferation of CTs is followed by differentiation of daughter cells that have left the cell cycle. Then, syncytial fusion of highly differentiated CTs with the overlying ST occurs. Within the ST, further differentiation and maturation takes place, leading to aging and late apoptosis of nuclei and other organelles at specific sites [261, 262]. Regulation of apoptotic processes is not only important for programmed morphogenesis, but also suggestively involved in syncytium formation, being of significant relevance to the biology of early placental villi in general [263]. The balance of the dynamics between apoptosis, proliferation and differentiation is a hallmark of developmental regulation and the key to normal development of the villous tree. Any high dimensional error in this balance may predispose villous growth to anomaly and/or insufficiency [264]. Hence, any changes that interfere with the delicate process of turnover of trophoblast cells may compromise pregnancy success and lead to some pregnancy-associated disorders. A large number of cytokines, growth factors and steroid hormones participate in these processes and endocannabinoids may be important players in this cytokine network.

During pregnancy, the placenta and the fetus become frequently exposed to drugs of abuse such as cannabinoids because of maternal consumption of these substances. This may cause disturbances on cannabinoid signaling and on the ECS homeostasis. Thus, the aim of this work was to explore the effects of phyto-, synthetic and endocannabinoids in cytotrophoblasts, to clarify the impact of cannabinoid compounds in placentation.

In this study the results showed that the main psychoactive compound of the cannabis plant, THC, did not affect BeWo cells viability. This phytocannabinoid did not induce the formation of reactive species of oxygen/nitrogen but caused a decrease in mitochondrial membrane potential. However, recently, our group reported that THC has a dual effect in primary cultures of human trophoblasts: enhancing MTT metabolism at low concentrations, whereas at higher doses decreased cell viability. The prevention of trophoblast cell death seems to result from an antioxidant effect, as showed by the decrease in the generation of ROS/RNS. Moreover, THC impaired CTs syncytialization [251]. The antioxidant properties of THC and other phytocannabinoids were also reported by Chen and Buck as THC prevented serum-deprived oxidative cell death in B lymphoblastoid cells and fibroblasts. [265]. On the other hand, Khare et al. reported that THC inhibits cell proliferation and activates a restricted tight transcriptional program in the BeWo cells, modulating genes encoding for cell morphology, growth and apoptosis [266]. THC ability to induce apoptosis

has been described in several types of cancer cells, as in the case of prostate PC-3 cells via a receptor-independent mechanism [267] and in melanoma cells, by activation of an autophagic-mediated apoptosis [193]. Besides the suggested interference with trophoblast turnover, during pregnancy, THC may also interfere with nutrient transport across the placenta. Keating et al. reported that chronic exposure to THC decreased folic acid uptake, a micronutrient that is particularly important during pregnancy for normal placental and fetal development [268].

The synthetic cannabinoids emerged in recent years as drugs of abuse and little is known about their effects in general and particularly in reproductive health. In this work, the synthetic cannabinoid WIN was able to induce a dose-dependent loss of cell viability that was evident at lower concentrations than the other studied cannabinoids. MTT results revealed that WIN at 2 µM was able to decrease cell viability and, at this concentration, no significant LDH release was detected, indicating that cell viability loss was not due to a necrotic process of cell death. To explore the WIN-induced loss of cell viability mechanisms, it was evaluated the mitochondrial membrane potential which was decreased, without the involvement of ROS/RNS production. This suggests that mitochondrial events might be involved. Indeed, this compound induced mitochondrial depolarization in other cell models, such as glioma cells [269] and hepatoma cells [270]. In glioma cells, WIN induced a down-regulation of Akt and Erk signaling, activation of caspases and a decrease in the levels of phosphorylated Bad [269]. In hepatoma HepG2 cells, it was also observed a mitochondrial depolarization accompanied by down-regulation of survival factors and, associated with JNK/p38 MAPK pathway activation [270]. Nevertheless, the observed loss of cell viability induced by the cannabinoids under study may be due to antiproliferative effects. In this way, cell cycle analysis was studied by flow cytometry. The results revealed a cell cycle arrest in G_0/G_1 after WIN treatment. These results are in accordance to the reports in other cell models. In human prostate cancer cells, treatment with WIN for 24 hours resulted in cell cycle arrest in the G_0/G_1 phase, which seems to be due to ERK1/2 activation, subsequently leading to an induction of apoptosis [271]. Park et al., in a study performed in gastric cancer cells, showed that WIN reduced cell proliferation, via G_0/G_1 phase cell cycle arrest, which preceded apoptosis and was mediated via activation of MAPK pathway and activation of pAKT [272]. In human breast cancer cells, WIN blocks tumor growth by inducing cell cycle arrest in G_0/G_1 and apoptosis through a CB-dependent manner [273].

The results of morphological analysis revealed that, after treatment with WIN, BeWo cells presented a marked cytoplasmic vacuolization and chromatin condensation. Notaro et al. also observed similar morphological alterations in osteosarcoma cells. In their study,

WIN induced a G_2/M phase cell cycle arrest, associated with the induction of the main markers of ER-stress (GRP78, CHOP and TRIB3), conversion of LC3-I into LC3-II and the enhanced incorporation of monodansylcadaverine and acridine orange, two markers of the autophagic compartments such as autolysosomes [197]. In a study performed in mantle cell lymphoma a massive vacuolization was also observed after WIN treatment but, in this case, the persistent expression of mammalian homolog of Atg8 with microtubule-associated LC3-II and p62, as well as the lack of protection from the autophagic inhibitor chloroquine, indicated that lysosomal degradation was not involved in this cytoplasmic vacuolization [274]. In decidual fibroblasts, stimulation of CB1 by WIN led to a decrease in intracellular cAMP levels and to an increase in DNA fragmentation and caspase-3 levels, suggesting a stimulation of apoptosis by this cannabinoid and an interference with the decidualization [275].

Depending on the system, though the majority of studies point to an antiproliferative effect of WIN, which is of great interest for the treatment of cancer, this compound can also have neuroprotective properties. In dentate gyrus granule cells, WIN showed to have neuroprotective properties, by acting upon CB1 receptors and, at higher concentrations, also other receptors were activated [276]. In our study, the effects were not reverted by the co-incubation with the CB1 and CB2 antagonists, AM251 and AM630, respectively. This observation suggest that WIN effects may be CB-independent and other signaling pathways can be involved.

Following previous works from the group, the effects of the endocannabinoid 2-AG were explored. Costa et al. have previously observed that this compound was able to decrease cell viability, inducing apoptosis in BeWo cells [129] and impair CTs syncytialization, interfering with pregnancy-related hormones expression, such as chorionic gonadotropin and leptin [238]. The results demonstrate that, in contrast with the other studied cannabinoids, 2-AG was able to increase ROS/RNS generation in both BeWo cells and hCTs, suggesting that the mechanism of 2-AG action may involve oxidative stress. This could be of great relevance, since placenta develops in a state of hypoxia, and any oxidative changes may disturb homeostasis. Moreover, mitochondrial membrane potential was also affected by 2-AG treatment, which could be associated with early apoptotic events. The effect on mitochondrial membrane potential was partially reversed by the addition of the CB2 antagonist AM630 in CT. In accordance with our findings, 2-AG also induced death of hepatic stellate cells via mitochondrial reactive oxygen species, accompanied by mitochondrial depolarization [277]. Accumulating evidence suggests that protein folding and generation of ROS as a byproduct of protein oxidation in the ER are closely linked events.

It has also become apparent that activation of the unfold protein response (a highly specific ER quality-control system) on exposure to oxidative stress is an adaptive mechanism to preserve cell function and survival. Persistent oxidative stress and protein misfolding initiate apoptotic cascades [278, 279]. CHOP is induced by ER-stress and causes downregulation of the anti-apoptotic protein Bcl-2, favoring a pro-apoptotic drive at the mitochondria by proteins that cause mitochondrial damage, cytochrome *c* release and caspase-3 activation [280]. To deeply explore ER-stress involvement in 2-AG actions, it was analyzed the expression of the ER-stress-related protein CHOP and it was observed an increase in CHOP expression after treatment. The co-incubation with AM630 reversed the effects on both BeWo and hCT cells, suggesting that 2-AG effects relies on CB2 activation. Moreover, the preliminary results in CTs indicate an activation of the executioner caspases-3/7, supporting an apoptotic process of cell death. Additionally, the analysis of DNA content by flow cytometry showed that 2-AG-treated cells presented an increase in the population in G_2/M phase, which was already described to be related with apoptosis [281].

Our results highlight the importance of eCBs in cytotrophoblast cell apoptosis and suggest that deregulation of ECS may interfere with normal trophoblat turnover.

Final conclusions

The work developed in the scope of this master's thesis compares the effects of three different types of cannabinoids in cytotrophoblast cells. The phytocannabinoid THC did not affect cell viability in the studied conditions, in contrast with the synthetic cannabinoid WIN that was able to decrease cell viability at low concentrations. No changes in the oxidative state were detected, but WIN induced mitochondrial membrane depolarization, cell cycle arrest in G_0/G_1 phase and morphological changes suggesting that autophagy may take part in the observed loss of cell viability. Following our previous results reporting 2-AG induced-apoptosis in BeWo cells it was observed a decrease in mitochondrial membrane potential associated to ROS/RNS generation. Endoplasmic reticulum stress may be involved, as preliminary results point to an increase in CHOP expression. In addition, 2-AG induced cell cycle alterations causing a G_2/M phase cell cycle arrest.

This work demonstrates that, in placenta, cannabinoids may interfere with important processes of cell proliferation and cell death. Understanding the effects of cannabinoid compounds in human reproduction, more specifically in women fertility is urgent, since cannabis and derivates are the most consumed illicit drugs worldwide and some of the most consumed recreational drugs by pregnant women. The consumption of such drugs may alter the ECS balance, ultimately interfering with eCB levels, and leading to pregnancy complications and infertility. So, the knowledge about ECS, cannabinoid signaling and cannabinoids effects in placental cells may help to disclose the cellular mechanisms behind some pregnancy-related disorders and ultimately be used to design future treatments for infertility.

References

References

1. Di Marzo, V. and L.D. Petrocellis, *Plant, synthetic, and endogenous cannabinoids in medicine.* Annu Rev Med, 2006. **57**: p. 553-74.

2. Pertwee, R.G., *Pharmacology of cannabinoid CB1 and CB2 receptors.* Pharmacol Ther, 1997. **74**(2): p. 129-80.

3. Herkenham, M., A.B. Lynn, M.R. Johnson, L.S. Melvin, B.R. de Costa, and K.C. Rice, *Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study.* J Neurosci, 1991. **11**(2): p. 563-83.

4. Griffin, G., Q. Tao, and M.E. Abood, *Cloning and pharmacological characterization of the rat CB(2) cannabinoid receptor.* J Pharmacol Exp Ther, 2000. **292**(3): p. 886-94.

5. Van Sickle, M.D., M. Duncan, P.J. Kingsley, A. Mouihate, P. Urbani, K. Mackie, N. Stella, A. Makriyannis, D. Piomelli, J.S. Davison, L.J. Marnett, V. Di Marzo, Q.J. Pittman, K.D. Patel, and K.A. Sharkey, *Identification and functional characterization of brainstem cannabinoid CB2 receptors.* Science, 2005. **310**(5746): p. 329-32.

6. Riehl, S., M. Zeidi, and N.J. Conard, *Emergence of agriculture in the foothills of the Zagros Mountains of Iran.* Science, 2013. **341**(6141): p. 65-7.

7. Touw, M., *The religious and medicinal uses of Cannabis in China, India and Tibet.* J Psychoactive Drugs, 1981. **13**(1): p. 23-34.

8. Fergusson, D.M., L.J. Horwood, and K. Northstone, *Maternal use of cannabis and pregnancy outcome.* Bjog, 2002. **109**(1): p. 21-7.

9. Global Drug Survey Data 2014; Available from: http://www.globaldrugsurvey.com/the-global-drug-survey-2014-findings/, accessed in 14 October 2015

10. Mechoulam, R. and Y. Gaoni, *A total synthesis of DL-DELTA-1-Tetrahydrocannabinol, the active constituent of hashish.* J Am Chem Soc, 1965. **87**: p. 3273-5.

11. Michoulam, R. and Y. Shvo, *Hashish. I. The structure of cannabidiol.* Tetrahedron, 1963. **19**(12): p. 2073-8.

12. Mehmedic, Z., S. Chandra, D. Slade, H. Denham, S. Foster, A.S. Patel, S.A. Ross, I.A. Khan, and M.A. ElSohly, *Potency trends of Delta9-THC and other cannabinoids in confiscated cannabis preparations from 1993 to 2008.* J Forensic Sci, 2010. **55**(5): p. 1209-17.

13. Hall, W., *U.S. Policy Responses to Calls for the Medical Use of Cannabis.* Yale J Biol Med, 2015. **88**(3): p. 257-64.

14. Tramer, M.R., D. Carroll, F.A. Campbell, D.J. Reynolds, R.A. Moore, and H.J. McQuay, *Cannabinoids for control of chemotherapy induced nausea and vomiting: quantitative systematic review.* British Medical Journal, 2001. **323**(7303): p. 16-21.

15. Lutge, E.E., A. Gray, and N. Siegfried, *The medical use of cannabis for reducing morbidity and mortality in patients with HIV/AIDS.* Cochrane Database Syst Rev, 2013. **4**:Cd005175.

16. Martin-Sanchez, E., T.A. Furukawa, J. Taylor, and J.L. Martin, *Systematic review and meta-analysis of cannabis treatment for chronic pain.* Pain Med, 2009. **10**(8): p. 1353-68.

17. Fife, T.D., H. Moawad, C. Moschonas, K. Shepard, and N. Hammond, *Clinical perspectives on medical marijuana (cannabis) for neurologic disorders.* Neurol Clin Pract, 2015. **5**(4): p. 344-351.

18. Devinsky, O., M.R. Cilio, H. Cross, J. Fernandez-Ruiz, J. French, C. Hill, R. Katz, V. Di Marzo, D. Jutras-Aswad, W.G. Notcutt, J. Martinez-Orgado, P.J. Robson, B.G. Rohrback, E. Thiele, B. Whalley, and D. Friedman, *Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders.* Epilepsia, 2014. **55**(6): p. 791-802.

19. Sun, X., C.S. Xu, N. Chadha, A. Chen, and J. Liu, *Marijuana for Glaucoma: A Recipe for Disaster or Treatment?* Yale J Biol Med, 2015. **88**(3): p. 265-9.

20. Reichenbach, Z.W., J. Sloan, A. Rizvi-Toner, L. Bayman, J. Valestin, and R. Schey, *A 4-Week Pilot Study With the Cannabinoid Receptor Agonist Dronabinol and Its Effect on Metabolic Parameters in a Randomized Trial.* Clin Ther, 2015. **37**(10): p. 2267-74.

21. Rahn, E.J. and A.G. Hohmann, *Cannabinoids as pharmacotherapies for neuropathic pain: from the bench to the bedside.* Neurotherapeutics, 2009. **6**(4): p. 713-37.

22. Leocani, L., A. Nuara, E. Houdayer, I. Schiavetti, U. Del Carro, S. Amadio, L. Straffi, P. Rossi, V. Martinelli, C. Vila, M.P. Sormani, and G. Comi, *Sativex and clinical-neurophysiological measures of spasticity in progressive multiple sclerosis.* J Neurol, 2015. **262**(11): p. 2520-7

23. Russo, E.B., *Cannabinoids in the management of difficult to treat pain.* Ther Clin Risk Manag, 2008. **4**(1): p. 245-59.

24. Seamon, M.J., J.A. Fass, M. Maniscalco-Feichtl, and N.A. Abu-Shraie, *Medical marijuana and the developing role of the pharmacist.* Am J Health Syst Pharm, 2007. **64**(10): p. 1037-44.

25. Radhakrishnan, R., S.T. Wilkinson, and D.C. D'Souza, *Gone to Pot - A Review of the Association between Cannabis and Psychosis.* Front Psychiatry, 2014. **5**:54.

26. Bovasso, G.B., *Cannabis abuse as a risk factor for depressive symptoms.* Am J Psychiatry, 2001. **158**(12): p. 2033-7.

27. Lutchmansingh, D., L. Pawar, and D. Savici, *Legalizing Cannabis: A physician's primer on the pulmonary effects of marijuana.* Curr Respir Care Rep, 2014. **3**(4): p. 200-205.

28. Passey, M.E., R.W. Sanson-Fisher, C.A. D'Este, and J.M. Stirling, *Tobacco, alcohol and cannabis use during pregnancy: clustering of risks.* Drug Alcohol Depend, 2014. **134**: p. 44-50.

29. Mills, B., A. Yepes, and K. Nugent, *Synthetic Cannabinoids.* Am J Med Sci, 2015. **350**(1): p. 59-62.

30. Downey, L.A. and J.C. Verster, *Cannabis concerns: increased potency, availability and synthetic analogues.* Curr Drug Abuse Rev, 2014. **7**(2): p. 67-8.

31. Vigolo, A., A. Ossato, C. Trapella, F. Vincenzi, C. Rimondo, C. Seri, K. Varani, G. Serpelloni, and M. Marti, *Novel halogenated derivates of JWH-018: Behavioral and binding studies in mice.* Neuropharmacology, 2015. **95**: p. 68-82.

32. Aung, M.M., G. Griffin, J.W. Huffman, M. Wu, C. Keel, B. Yang, V.M. Showalter, M.E. Abood, and B.R. Martin, *Influence of the N-1 alkyl chain length of cannabimimetic indoles upon CB(1) and CB(2) receptor binding.* Drug Alcohol Depend, 2000. **60**(2): p. 133-40.

33. Zawilska, J.B., *"Legal Highs"--An Emerging Epidemic of Novel Psychoactive Substances.* Int Rev Neurobiol, 2015. **120**: p. 273-300.

34. Global Drug Survey Data 2015; Available from: http://www.globaldrugsurvey.com/the-global-drug-survey-2015-findings/, accessed in 14 October 2015.

35. Carroll, F.I., A.H. Lewin, S.W. Mascarella, H.H. Seltzman, and P.A. Reddy, *Designer drugs: a medicinal chemistry perspective.* Ann N Y Acad Sci, 2012. **1248**: p. 18-38.

36. Elsohly, M.A., W. Gul, A.S. Wanas, and M.M. Radwan, *Synthetic cannabinoids: analysis and metabolites.* Life Sci, 2014. **97**(1): p. 78-90.

37. Wiley, J.L., J.A. Marusich, and J.W. Huffman, *Moving around the molecule: relationship between chemical structure and in vivo activity of synthetic cannabinoids.* Life Sci, 2014. **97**(1): p. 55-63.

38. Pop, E., *Cannabinoids, endogenous ligands and synthetic analogs.* Curr Opin Chem Biol, 1999. **3**(4): p. 418-25.

39. Citraro, R., E. Russo, R.T. Ngomba, F. Nicoletti, F. Scicchitano, B.J. Whalley, A. Calignano, and G. De Sarro, *CB1 agonists, locally applied to the cortico-thalamic circuit of rats with genetic absence epilepsy, reduce epileptic manifestations.* Epilepsy Res, 2013. **106**(1-2): p. 74-82.

40. Vilela, L.R., D.C. Medeiros, G.H. Rezende, A.C. de Oliveira, M.F. Moraes, and F.A. Moreira, *Effects of cannabinoids and endocannabinoid hydrolysis inhibition on pentylenetetrazole-induced seizure and electroencephalographic activity in rats.* Epilepsy Res, 2013. **104**(3): p. 195-202.

41. Colin-Gonzalez, A.L., A.L. Paz-Loyola, I.N. Serratos, B. Seminotti, C.A. Ribeiro, G. Leipnitz, D.O. Souza, M. Wajner, and A. Santamaria, *The effect of WIN 55,212-2 suggests a cannabinoid-sensitive component in the early toxicity induced by organic acids accumulating in glutaric acidemia type I and in related disorders of propionate metabolism in rat brain synaptosomes.* Neuroscience, 2015. **310**: p. 578-88.

42. Gingold, A.R. and N.V. Bergasa, *The cannabinoid agonist WIN 55, 212-2 increases nociception threshold in cholestatic rats: implications for the treatment of the pruritus of cholestasis.* Life Sci, 2003. **73**(21): p. 2741-7.

43. Potenzieri, C., C. Harding-Rose, and D.A. Simone, *The cannabinoid receptor agonist, WIN 55, 212-2, attenuates tumor-evoked hyperalgesia through peripheral mechanisms.* Brain Res, 2008. **1215**: p. 69-75.

44. Dunn, S.L., J.M. Wilkinson, A. Crawford, C.L. Le Maitre, and R.A. Bunning, *Cannabinoid WIN-55,212-2 mesylate inhibits interleukin-1beta induced matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase expression in human chondrocytes.* Osteoarthritis Cartilage, 2014. **22**(1): p. 133-44.

45. Devane, W.A., L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam, *Isolation and structure of a brain constituent that binds to the cannabinoid receptor.* Science, 1992. **258**(5090): p. 1946-9.

46. Munro, S., K.L. Thomas, and M. Abu-Shaar, *Molecular characterization of a peripheral receptor for cannabinoids.* Nature, 1993. **365**(6441): p. 61-5.

47. Mechoulam, R., S. Ben-Shabat, L. Hanus, M. Ligumsky, N.E. Kaminski, A.R. Schatz, A. Gopher, S. Almog, B.R. Martin, D.R. Compton, and et al., *Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors.* Biochem Pharmacol, 1995. **50**(1): p. 83-90.

48. Hanus, L., S. Abu-Lafi, E. Fride, A. Breuer, Z. Vogel, D.E. Shalev, I. Kustanovich, and R. Mechoulam, *2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 3662-5.

49. Porter, A.C., J.M. Sauer, M.D. Knierman, G.W. Becker, M.J. Berna, J. Bao, G.G. Nomikos, P. Carter, F.P. Bymaster, A.B. Leese, and C.C. Felder, *Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor.* J Pharmacol Exp Ther, 2002. **301**(3): p. 1020-4.

50. Bisogno, T., D. Melck, M. Bobrov, N.M. Gretskaya, V.V. Bezuglov, L. De Petrocellis, and V. Di Marzo, *N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo.* Biochem J, 2000. **351 Pt 3**: p. 817-24.

51. Cascio, M.G. and P. Marini, *Biosynthesis and Fate of Endocannabinoids.* Handb Exp Pharmacol, 2015. **231**: p. 39-58.

52. Lambert, D.M. and V. Di Marzo, *The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic?* Curr Med Chem, 1999. **6**(8): p. 757-73.

53. Hansen, H.S., B. Moesgaard, H.H. Hansen, and G. Petersen, *N-Acylethanolamines and precursor phospholipids - relation to cell injury.* Chem Phys Lipids, 2000. **108**(1-2): p. 135-50.

54. Fonseca, B.M., M.A. Costa, M. Almada, G. Correia-da-Silva, and N.A. Teixeira, *Endogenous cannabinoids revisited: a biochemistry perspective.* Prostaglandins Other Lipid Mediat, 2013. **102-103**: p. 13-30.

55. Maccarrone, M. and A. Finazzi-Agro, *The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis.* Cell Death Differ, 2003. **10**(9): p. 946-55.

56. De Petrocellis, L. and V. Di Marzo, *Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels.* J Neuroimmune Pharmacol, 2010. **5**(1): p. 103-21.

57. Toth, A., P.M. Blumberg, and J. Boczan, *Anandamide and the vanilloid receptor (TRPV1).* Vitam Horm, 2009. **81**: p. 389-419.

58. Sun, Y., S.P. Alexander, D.A. Kendall, and A.J. Bennett, *Cannabinoids and PPARalpha signalling.* Biochem Soc Trans, 2006. **34**(Pt 6): p. 1095-7.

59. Bouaboula, M., S. Hilairet, J. Marchand, L. Fajas, G. Le Fur, and P. Casellas, *Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation.* Eur J Pharmacol, 2005. **517**(3): p. 174-81.

60. Maccarrone, M., E. Dainese, and S. Oddi, *Intracellular trafficking of anandamide: new concepts for signaling.* Trends Biochem Sci, 2010. **35**(11): p. 601-8.

61. Smita, K., V. Sushil Kumar, and J.S. Premendran, *Anandamide: an update.* Fundam Clin Pharmacol, 2007. **21**(1): p. 1-8.

62. Sugiura, T., *Physiological roles of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand.* Biofactors, 2009. **35**(1): p. 88-97.

63. Campos, A.C., F.A. Moreira, F.V. Gomes, E.A. Del Bel, and F.S. Guimaraes, *Multiple mechanisms involved in the large-spectrum therapeutic potential of*

cannabidiol in psychiatric disorders. Philos Trans R Soc Lond B Biol Sci, 2012. **367**(1607): p. 3364-78.

64. Osei-Hyiaman, D., M. DePetrillo, P. Pacher, J. Liu, S. Radaeva, S. Batkai, J. Harvey-White, K. Mackie, L. Offertaler, L. Wang, and G. Kunos, *Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity.* J Clin Invest, 2005. **115**(5): p. 1298-305.

65. Maresz, K., G. Pryce, E.D. Ponomarev, G. Marsicano, J.L. Croxford, L.P. Shriver, C. Ledent, X. Cheng, E.J. Carrier, M.K. Mann, G. Giovannoni, R.G. Pertwee, T. Yamamura, N.E. Buckley, C.J. Hillard, B. Lutz, D. Baker, and B.N. Dittel, *Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells.* Nat Med, 2007. **13**(4): p. 492-7.

66. Woodhams, S.G., D.R. Sagar, J.J. Burston, and V. Chapman, *The role of the endocannabinoid system in pain.* Handb Exp Pharmacol, 2015. **227**: p. 119-43.

67. Boorman, E., Z. Zajkowska, R. Ahmed, C.M. Pariante, and P.A. Zunszain, *Crosstalk between endocannabinoid and immune systems: a potential dysregulation in depression?* Psychopharmacology (Berl), 2015. **(ahead of print)**.

68. Battista, N., M. Bari, and M. Maccarrone, *Endocannabinoids and Reproductive Events in Health and Disease.* Handb Exp Pharmacol, 2015. **231**: p. 341-65.

69. O'Sullivan, S.E., *Endocannabinoids and the Cardiovascular System in Health and Disease.* Handb Exp Pharmacol, 2015. **231**: p. 393-422.

70. B.M. Fonseca, M.A.C., M. Almada, A. Soares, G. Correia-da-Silva, Natércia A. Teixeira, *O Sistema Endocanabinóide – uma perspetiva terapêutica.* Acta Farmacêutica Portuguesa, 2013. **2**(2): p. 37-44.

71. Simon, E.J., *In search of the opiate receptor.* Am J Med Sci, 1973. **266**(3): p. 160-8.

72. Devane, W.A., F.A. Dysarz, 3rd, M.R. Johnson, L.S. Melvin, and A.C. Howlett, *Determination and characterization of a cannabinoid receptor in rat brain.* Mol Pharmacol, 1988. **34**(5): p. 605-13.

73. Matsuda, L.A., S.J. Lolait, M.J. Brownstein, A.C. Young, and T.I. Bonner, *Structure of a cannabinoid receptor and functional expression of the cloned cDNA.* Nature, 1990. **346**(6284): p. 561-4.

74. Matsuda, L.A., T.I. Bonner, and S.J. Lolait, *Localization of cannabinoid receptor mRNA in rat brain.* J Comp Neurol, 1993. **327**(4): p. 535-50.

75. Pertwee, R.G., A.C. Howlett, M.E. Abood, S.P. Alexander, V. Di Marzo, M.R. Elphick, P.J. Greasley, H.S. Hansen, G. Kunos, K. Mackie, R. Mechoulam, and R.A. Ross, *International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2).* Pharmacol Rev, 2010. **62**(4): p. 588-631.

76. Dainese, E., S. Oddi, M. Bari, and M. Maccarrone, *Modulation of the endocannabinoid system by lipid rafts.* Curr Med Chem, 2007. **14**(25): p. 2702-15.

77. Howlett, A.C., J.M. Qualy, and L.L. Khachatrian, *Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs.* Mol Pharmacol, 1986. **29**(3): p. 307-13.

78. Felder, C.C., K.E. Joyce, E.M. Briley, J. Mansouri, K. Mackie, O. Blond, Y. Lai, A.L. Ma, and R.L. Mitchell, *Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors.* Mol Pharmacol, 1995. **48**(3): p. 443-50.

79. Howlett, A.C., *Cannabinoid receptor signaling.* Handb Exp Pharmacol, 2005(168): p. 53-79.

80. Glass, M. and C.C. Felder, *Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor.* J Neurosci, 1997. **17**(14): p. 5327-33.

81. McIntosh, B.T., B. Hudson, S. Yegorova, C.A. Jollimore, and M.E. Kelly, *Agonist-dependent cannabinoid receptor signalling in human trabecular meshwork cells.* Br J Pharmacol, 2007. **152**(7): p. 1111-20.

82. Mu, J., S.Y. Zhuang, R.E. Hampson, and S.A. Deadwyler, *Protein kinase-dependent phosphorylation and cannabinoid receptor modulation of potassium A current (IA) in cultured rat hippocampal neurons.* Pflugers Arch, 2000. **439**(5): p. 541-6.

83. Derkinderen, P., M. Toutant, F. Burgaya, M. Le Bert, J.C. Siciliano, V. de Franciscis, M. Gelman, and J.A. Girault, *Regulation of a neuronal form of focal adhesion kinase by anandamide.* Science, 1996. **273**(5282): p. 1719-22.

84. Zhou, D. and Z.H. Song, *CB1 cannabinoid receptor-mediated tyrosine phosphorylation of focal adhesion kinase-related non-kinase.* FEBS Lett, 2002. **525**(1-3): p. 164-8.

85. Rhee, M.H., M. Bayewitch, T. Avidor-Reiss, R. Levy, and Z. Vogel, *Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes.* J Neurochem, 1998. **71**(4): p. 1525-34.

86. Bouaboula, M., C. Poinot-Chazel, B. Bourrie, X. Canat, B. Calandra, M. Rinaldi-Carmona, G. Le Fur, and P. Casellas, *Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1.* Biochem J, 1995. **312 (Pt 2)**: p. 637-41.

87. Rueda, D., I. Galve-Roperh, A. Haro, and M. Guzman, *The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase.* Mol Pharmacol, 2000. **58**(4): p. 814-20.

88. Velasco, G., I. Galve-Roperh, C. Sanchez, C. Blazquez, A. Haro, and M. Guzman, *Cannabinoids and ceramide: two lipids acting hand-by-hand.* Life Sci, 2005. **77**(14): p. 1723-31.

89. Arold, S.T., *How focal adhesion kinase achieves regulation by linking ligand binding, localization and action.* Curr Opin Struct Biol, 2011. **21**(6): p. 808-13.

90. Guzman, M., C. Sanchez, and I. Galve-Roperh, *Control of the cell survival/death decision by cannabinoids.* J Mol Med (Berl), 2001. **78**(11): p. 613-25.

91. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.

92. Shalini, S., L. Dorstyn, S. Dawar, and S. Kumar, *Old, new and emerging functions of caspases.* Cell Death Differ, 2015. **22**(4): p. 526-39.

93. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.

94. Slee, E.A., C. Adrain, and S.J. Martin, *Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis.* J Biol Chem, 2001. **276**(10): p. 7320-6.

95. Sakahira, H., M. Enari, and S. Nagata, *Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.* Nature, 1998. **391**(6662): p. 96-9.

96. Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, and L.T. Williams, *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis.* Science, 1997. **278**(5336): p. 294-8.

97. Bratton, D.L., V.A. Fadok, D.A. Richter, J.M. Kailey, L.A. Guthrie, and P.M. Henson, *Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase.* J Biol Chem, 1997. **272**(42): p. 26159-65.

98. de Almagro, M.C. and D. Vucic, *The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy.* Exp Oncol, 2012. **34**(3): p. 200-11.

99. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation.* Science, 1998. **281**(5381): p. 1305-8.

100. Hsu, H., J. Xiong, and D.V. Goeddel, *The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation.* Cell, 1995. **81**(4): p. 495-504.

101. Wajant, H., *The Fas signaling pathway: more than a paradigm.* Science, 2002. **296**(5573): p. 1635-6.

102. Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Krammer, and M.E. Peter, *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor.* Embo j, 1995. **14**(22): p. 5579-88.

103. Scaffidi, C., I. Schmitz, P.H. Krammer, and M.E. Peter, *The role of c-FLIP in modulation of CD95-induced apoptosis.* J Biol Chem, 1999. **274**(3): p. 1541-8.

104. Saelens, X., N. Festjens, L. Vande Walle, M. van Gurp, G. van Loo, and P. Vandenabeele, *Toxic proteins released from mitochondria in cell death.* Oncogene, 2004. **23**(16): p. 2861-74.

105. Hill, M.M., C. Adrain, P.J. Duriez, E.M. Creagh, and S.J. Martin, *Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes.* Embo j, 2004. **23**(10): p. 2134-45.

106. Schimmer, A.D., *Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice.* Cancer Res, 2004. **64**(20): p. 7183-90.

107. Li, L.Y., X. Luo, and X. Wang, *Endonuclease G is an apoptotic DNase when released from mitochondria.* Nature, 2001. **412**(6842): p. 95-9.

108. Joza, N., S.A. Susin, E. Daugas, W.L. Stanford, S.K. Cho, C.Y. Li, T. Sasaki, A.J. Elia, H.Y. Cheng, L. Ravagnan, K.F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y.Y. Kong, T.W. Mak, J.C. Zuniga-Pflucker, G. Kroemer, and J.M. Penninger, *Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death.* Nature, 2001. **410**(6828): p. 549-54.

109. Susin, S.A., E. Daugas, L. Ravagnan, K. Samejima, N. Zamzami, M. Loeffler, P. Costantini, K.F. Ferri, T. Irinopoulou, M.C. Prevost, G. Brothers, T.W. Mak, J. Penninger, W.C. Earnshaw, and G. Kroemer, *Two distinct pathways leading to nuclear apoptosis.* J Exp Med, 2000. **192**(4): p. 571-80.

110. Doerflinger, M., J.A. Glab, and H. Puthalakath, *BH3-only proteins: a 20-year stock-take.* Febs j, 2015. **282**(6): p. 1006-16.

111. Billen, L.P., A. Shamas-Din, and D.W. Andrews, *Bid: a Bax-like BH3 protein.* Oncogene, 2008. **27 Suppl 1**: p. S93-104.

112. Sanchez, C., I. Galve-Roperh, C. Canova, P. Brachet, and M. Guzman, *Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells.* FEBS Lett, 1998. **436**(1): p. 6-10.

113. Sarker, K.P., S. Obara, M. Nakata, I. Kitajima, and I. Maruyama, *Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3.* FEBS Lett, 2000. **472**(1): p. 39-44.

114. Maccarrone, M., T. Lorenzon, M. Bari, G. Melino, and A. Finazzi-Agro, *Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors.* J Biol Chem, 2000. **275**(41): p. 31938-45.

115. Chan, G.C., T.R. Hinds, S. Impey, and D.R. Storm, *Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol.* J Neurosci, 1998. **18**(14): p. 5322-32.

116. Galve-Roperh, I., C. Sanchez, M.L. Cortes, T. Gomez del Pulgar, M. Izquierdo, and M. Guzman, *Anti-tumoral action of cannabinoids: involvement of sustained*

ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med, 2000. **6**(3): p. 313-9.

117. Goswami, R. and G. Dawson, *Does ceramide play a role in neural cell apoptosis?* J Neurosci Res, 2000. **60**(2): p. 141-9.

118. Gomez del Pulgar, T., G. Velasco, C. Sanchez, A. Haro, and M. Guzman, *De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis.* Biochem J, 2002. **363**(Pt 1): p. 183-8.

119. Mimeault, M., N. Pommery, N. Wattez, C. Bailly, and J.P. Henichart, *Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: implication of epidermal growth factor receptor down-regulation and ceramide production.* Prostate, 2003. **56**(1): p. 1-12.

120. Tomiyama, K. and M. Funada, *Cytotoxicity of synthetic cannabinoids on primary neuronal cells of the forebrain: the involvement of cannabinoid CB1 receptors and apoptotic cell death.* Toxicol Appl Pharmacol, 2014. **274**(1): p. 17-23.

121. Hong, Y., Y. Zhou, Y. Wang, S. Xiao, D.J. Liao, and Q. Zhao, *PPARgamma mediates the effects of WIN55,212-2, an synthetic cannabinoid, on the proliferation and apoptosis of the BEL-7402 hepatocarcinoma cells.* Mol Biol Rep, 2013. **40**(11): p. 6287-93.

122. Soliman, E., K.L. Henderson, A.S. Danell, and R. Van Dross, *Arachidonoyl-ethanolamide activates endoplasmic reticulum stress-apoptosis in tumorigenic keratinocytes: Role of cyclooxygenase-2 and novel J-series prostamides.* Mol Carcinog, 2015 **(ahead of print).**

123. Adinolfi, B., A. Romanini, A. Vanni, E. Martinotti, A. Chicca, S. Fogli, and P. Nieri, *Anticancer activity of anandamide in human cutaneous melanoma cells.* Eur J Pharmacol, 2013. **718**(1-3): p. 154-9.

124. Borrelli, F., E. Pagano, B. Romano, S. Panzera, F. Maiello, D. Coppola, L. De Petrocellis, L. Buono, P. Orlando, and A.A. Izzo, *Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a Cannabis-derived non-psychotropic cannabinoid.* Carcinogenesis, 2014. **35**(12): p. 2787-97.

125. Morelli, M.B., M. Offidani, F. Alesiani, G. Discepoli, S. Liberati, A. Olivieri, M. Santoni, G. Santoni, P. Leoni, and M. Nabissi, *The effects of cannabidiol and its*

synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2. Int J Cancer, 2014. **134**(11): p. 2534-46.

126. Gomez, R., J. Conde, M. Scotece, V. Lopez, F. Lago, J.J. Gomez Reino, and O. Gualillo, *Endogenous cannabinoid anandamide impairs cell growth and induces apoptosis in chondrocytes.* J Orthop Res, 2014. **32**(9): p. 1137-46.

127. Fonseca, B.M., G. Correia-da-Silva, and N.A. Teixeira, *The endocannabinoid anandamide induces apoptosis of rat decidual cells through a mechanism involving ceramide synthesis and p38 MAPK activation.* Apoptosis, 2013. **18**(12): p. 1526-35.

128. Costa, M.A., B.M. Fonseca, N.A. Teixeira, and G. Correia-da-Silva, *The endocannabinoid anandamide induces apoptosis in cytotrophoblast cells: involvement of both mitochondrial and death receptor pathways.* Placenta, 2015. **36**(1): p. 69-76.

129. Costa, M.A., B.M. Fonseca, E. Keating, N.A. Teixeira, and G. Correia-da-Silva, *2-arachidonoylglycerol effects in cytotrophoblasts: metabolic enzymes expression and apoptosis in BeWo cells.* Reproduction, 2014. **147**(3): p. 301-11.

130. Jin, S., *Autophagy, mitochondrial quality control, and oncogenesis.* Autophagy, 2006. **2**(2): p. 80-4.

131. Lee, C.Y. and E.H. Baehrecke, *Steroid regulation of autophagic programmed cell death during development.* Development, 2001. **128**(8): p. 1443-55.

132. Baehrecke, E.H., *Autophagic programmed cell death in Drosophila.* Cell Death Differ, 2003. **10**(9): p. 940-5.

133. Berry, D.L. and E.H. Baehrecke, *Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila.* Cell, 2007. **131**(6): p. 1137-48.

134. Liu, Y. and B. Levine, *Autosis and autophagic cell death: the dark side of autophagy.* Cell Death Differ, 2015. **22**(3): p. 367-76.

135. Nelson, C. and E.H. Baehrecke, *Eaten to death.* Febs j, 2014. **281**(24): p. 5411-7.

136. Li, W.W., J. Li, and J.K. Bao, *Microautophagy: lesser-known self-eating.* Cell Mol Life Sci, 2012. **69**(7): p. 1125-36.

137. Klionsky, D.J., *The molecular machinery of autophagy: unanswered questions.* J Cell Sci, 2005. **118**(Pt 1): p. 7-18.

138. Yang, Z. and D.J. Klionsky, *Eaten alive: a history of macroautophagy.* Nat Cell Biol, 2010. **12**(9): p. 814-22.

139. Benbrook, D.M. and A. Long, *Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis.* Exp Oncol, 2012. **34**(3): p. 286-97.

140. Yoshii, S.R. and N. Mizushima, *Autophagy machinery in the context of mammalian mitophagy.* Biochim Biophys Acta, 2015. **1853**(10 Pt B): p.2797-801

141. Lemasters, J.J., *Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging.* Rejuvenation Res, 2005. **8**(1): p. 3-5.

142. Sakai, Y., M. Oku, I.J. van der Klei, and J.A. Kiel, *Pexophagy: autophagic degradation of peroxisomes.* Biochim Biophys Acta, 2006. **1763**(12): p. 1767-75.

143. Tuttle, D.L., A.S. Lewin, and W.A. Dunn, Jr., *Selective autophagy of peroxisomes in methylotrophic yeasts.* Eur J Cell Biol, 1993. **60**(2): p. 283-90.

144. Wang, F. and S. Muller, *Manipulating autophagic processes in autoimmune diseases: a special focus on modulating chaperone-mediated autophagy, an emerging therapeutic target.* Front Immunol, 2015. **6**:252.

145. Neff, N.T., L. Bourret, P. Miao, and J.F. Dice, *Degradation of proteins microinjected into IMR-90 human diploid fibroblasts.* J Cell Biol, 1981. **91**(1): p. 184-94.

146. Khaminets, A., T. Heinrich, M. Mari, P. Grumati, A.K. Huebner, M. Akutsu, L. Liebmann, A. Stolz, S. Nietzsche, N. Koch, M. Mauthe, I. Katona, B. Qualmann, J. Weis, F. Reggiori, I. Kurth, C.A. Hubner, and I. Dikic, *Regulation of endoplasmic reticulum turnover by selective autophagy.* Nature, 2015. **522**(7556): p. 354-8.

147. Mochida, K., Y. Oikawa, Y. Kimura, H. Kirisako, H. Hirano, Y. Ohsumi, and H. Nakatogawa, *Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus.* Nature, 2015. **522**(7556): p. 359-62.

148. Terman, A., B. Gustafsson, and U.T. Brunk, *Autophagy, organelles and ageing.* J Pathol, 2007. **211**(2): p. 134-43.

149. Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi, *Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction.* J Cell Biol, 1992. **119**(2): p. 301-11.

150. Thumm, M., R. Egner, B. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D.H. Wolf, *Isolation of autophagocytosis mutants of Saccharomyces cerevisiae.* FEBS Lett, 1994. **349**(2): p. 275-80.

151. Tsukada, M. and Y. Ohsumi, *Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae.* FEBS Lett, 1993. **333**(1-2): p. 169-74.

152. Chen, N. and V. Karantza-Wadsworth, *Role and regulation of autophagy in cancer.* Biochim Biophys Acta, 2009. **1793**(9): p. 1516-23.

153. Geng, J., M. Baba, U. Nair, and D.J. Klionsky, *Quantitative analysis of autophagy-related protein stoichiometry by fluorescence microscopy.* J Cell Biol, 2008. **182**(1): p. 129-40.

154. Suzuki, K. and Y. Ohsumi, *Current knowledge of the pre-autophagosomal structure (PAS).* FEBS Lett, 2010. **584**(7): p. 1280-6.

155. Corcelle, E.A., P. Puustinen, and M. Jaattela, *Apoptosis and autophagy: Targeting autophagy signalling in cancer cells -'trick or treats'?* Febs j, 2009. **276**(21): p. 6084-96.

156. Axe, E.L., S.A. Walker, M. Manifava, P. Chandra, H.L. Roderick, A. Habermann, G. Griffiths, and N.T. Ktistakis, *Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum.* The Journal of Cell Biology, 2008. **182**(4): p. 685-701.

157. Simonsen, A. and S.A. Tooze, *Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes.* J Cell Biol, 2009. **186**(6): p. 773-82.

158. Ylä-Anttila, P., H. Vihinen, E. Jokitalo, and E.-L. Eskelinen, *3D tomography reveals connections between the phagophore and endoplasmic reticulum.* Autophagy, 2014. **5**(8): p. 1180-85.

159. Mizushima, N. and D.J. Klionsky, *Protein turnover via autophagy: implications for metabolism.* Annu Rev Nutr, 2007. **27**: p. 19-40.

160. Rosenfeldt, M.T. and K.M. Ryan, *The role of autophagy in tumour development and cancer therapy.* Expert Rev Mol Med, 2009. **11**: e36.

161. Liang, X.H., S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, and B. Levine, *Induction of autophagy and inhibition of tumorigenesis by beclin 1.* Nature, 1999. **402**(6762): p. 672-6.

162. Eisenberg-Lerner, A. and A. Kimchi, *The paradox of autophagy and its implication in cancer etiology and therapy.* Apoptosis, 2009. **14**(4): p. 376-91.

163. Uchiyama, Y., M. Shibata, M. Koike, K. Yoshimura, and M. Sasaki, *Autophagy-physiology and pathophysiology.* Histochem Cell Biol, 2008. **129**(4): p. 407-20.

164. Ichimura, Y., T. Kirisako, T. Takao, Y. Satomi, Y. Shimonishi, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, T. Noda, and Y. Ohsumi, *A ubiquitin-like system mediates protein lipidation.* Nature, 2000. **408**(6811): p. 488-92.

165. Glick, D., S. Barth, and K.F. Macleod, *Autophagy: cellular and molecular mechanisms.* J Pathol, 2010. **221**(1): p. 3-12.

166. Pattingre, S., A. Tassa, X. Qu, R. Garuti, X.H. Liang, N. Mizushima, M. Packer, M.D. Schneider, and B. Levine, *Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy.* Cell, 2005. **122**(6): p. 927-39.

167. Moretti, L., E.S. Yang, K.W. Kim, and B. Lu, *Autophagy signaling in cancer and its potential as novel target to improve anticancer therapy.* Drug Resist Updat, 2007. **10**(4-5): p. 135-43.

168. Liang, C., P. Feng, B. Ku, I. Dotan, D. Canaani, B.H. Oh, and J.U. Jung, *Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG.* Nat Cell Biol, 2006. **8**(7): p. 688-99.

169. Takahashi, Y., D. Coppola, N. Matsushita, H.D. Cualing, M. Sun, Y. Sato, C. Liang, J.U. Jung, J.Q. Cheng, J.J. Mule, W.J. Pledger, and H.G. Wang, *Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis.* Nat Cell Biol, 2007. **9**(10): p. 1142-51.

170. Fimia, G.M., A. Stoykova, A. Romagnoli, L. Giunta, S. Di Bartolomeo, R. Nardacci, M. Corazzari, C. Fuoco, A. Ucar, P. Schwartz, P. Gruss, M. Piacentini, K. Chowdhury, and F. Cecconi, *Ambra1 regulates autophagy and development of the nervous system.* Nature, 2007. **447**(7148): p. 1121-5.

171. Tabancay, A.P., Jr., C.L. Gau, I.M. Machado, E.J. Uhlmann, D.H. Gutmann, L. Guo, and F. Tamanoi, *Identification of dominant negative mutants of Rheb GTPase and their use to implicate the involvement of human Rheb in the activation of p70S6K.* J Biol Chem, 2003. **278**(41): p. 39921-30.

172. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 21-35.

173. Kamada, Y., K. Yoshino, C. Kondo, T. Kawamata, N. Oshiro, K. Yonezawa, and Y. Ohsumi, *Tor directly controls the Atg1 kinase complex to regulate autophagy.* Mol Cell Biol, 2010. **30**(4): p. 1049-58.

174. Meijer, A.J. and P. Codogno, *AMP-activated protein kinase and autophagy.* Autophagy, 2007. **3**(3): p. 238-40.

175. Gingras, A.C., S.G. Kennedy, M.A. O'Leary, N. Sonenberg, and N. Hay, *4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway.* Genes Dev, 1998. **12**(4): p. 502-13.

176. Chang, Y.Y., G. Juhasz, P. Goraksha-Hicks, A.M. Arsham, D.R. Mallin, L.K. Muller, and T.P. Neufeld, *Nutrient-dependent regulation of autophagy through the target of rapamycin pathway.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 232-6.

177. Shaw, R.J., *LKB1 and AMP-activated protein kinase control of mTOR signalling and growth.* Acta Physiol (Oxf), 2009. **196**(1): p. 65-80.

178. Kim, J., M. Kundu, B. Viollet, and K.L. Guan, *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.

179. Kroemer, G., L. Galluzzi, and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiol Rev, 2007. **87**(1): p. 99-163.

180. Piyanova, A., O. Albayram, C.A. Rossi, H. Farwanah, K. Michel, P. Nicotera, K. Sandhoff, and A. Bilkei-Gorzo, *Loss of CB1 receptors leads to decreased cathepsin D levels and accelerated lipofuscin accumulation in the hippocampus.* Mech Ageing Dev, 2013. **134**(9): p. 391-9.

181. Shao, B.Z., W. Wei, P. Ke, Z.Q. Xu, J.X. Zhou, and C. Liu, *Activating cannabinoid receptor 2 alleviates pathogenesis of experimental autoimmune encephalomyelitis via activation of autophagy and inhibiting NLRP3 inflammasome.* CNS Neurosci Ther, 2014. **20**(12): p. 1021-8.

182. Casarejos, M.J., J. Perucho, A. Gomez, M.P. Munoz, M. Fernandez-Estevez, O. Sagredo, J. Fernandez Ruiz, M. Guzman, J.G. de Yebenes, and M.A. Mena, *Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy.* J Alzheimers Dis, 2013. **35**(3): p. 525-39.

183. Yang, L., R. Rozenfeld, D. Wu, L.A. Devi, Z. Zhang, and A. Cederbaum, *Cannabidiol protects liver from binge alcohol-induced steatosis by mechanisms including inhibition of oxidative stress and increase in autophagy.* Free Radic Biol Med, 2014. **68**: p. 260-7.

184. Oh, H.A., S. Kwon, S. Choi, H. Shin, K.H. Yoon, W.J. Kim, and H.J. Lim, *Uncovering a role for endocannabinoid signaling in autophagy in preimplantation mouse embryos.* Mol Hum Reprod, 2013. **19**(2): p. 93-101.

185. Scarlatti, F., R. Maffei, I. Beau, R. Ghidoni, and P. Codogno, *Non-canonical autophagy: an exception or an underestimated form of autophagy?* Autophagy, 2008. **4**(8): p. 1083-5.

186. Koay, L.C., R.J. Rigby, and K.L. Wright, *Cannabinoid-induced autophagy regulates suppressor of cytokine signaling-3 in intestinal epithelium.* Am J Physiol Gastrointest Liver Physiol, 2014. **307**(2): p. G140-8.

187. Salazar, M., A. Carracedo, Í.J. Salanueva, S. Hernández-Tiedra, M. Lorente, A. Egia, P. Vázquez, C. Blázquez, S. Torres, S. García, J. Nowak, G.M. Fimia, M. Piacentini, F. Cecconi, P.P. Pandolfi, L. González-Feria, J.L. Iovanna, M. Guzmán, P. Boya, and G. Velasco, *Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells.* Journal of Clinical Investigation, 2009. **119**(5): p. 1359-1372.

188. Chen, S., G. Bu, Y. Takei, K. Sakamoto, S. Ikematsu, T. Muramatsu, and K. Kadomatsu, *Midkine and LDL-receptor-related protein 1 contribute to the anchorage-independent cell growth of cancer cells.* J Cell Sci, 2007. **120**(Pt 22): p. 4009-15.

189. Mirkin, B.L., S. Clark, X. Zheng, F. Chu, B.D. White, M. Greene, and A. Rebbaa, *Identification of midkine as a mediator for intercellular transfer of drug resistance.* Oncogene, 2005. **24**(31): p. 4965-74.

190. Lorente, M., S. Torres, M. Salazar, A. Carracedo, S. Hernandez-Tiedra, F. Rodriguez-Fornes, E. Garcia-Taboada, B. Melendez, M. Mollejo, Y. Campos-Martin,

S.A. Lakatosh, J. Barcia, M. Guzman, and G. Velasco, *Stimulation of the midkine/ALK axis renders glioma cells resistant to cannabinoid antitumoral action.* Cell Death Differ, 2011. **18**(6): p. 959-73.

191. Shrivastava, A., P.M. Kuzontkoski, J.E. Groopman, and A. Prasad, *Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy.* Mol Cancer Ther, 2011. **10**(7): p. 1161-72.

192. Nabissi, M., M.B. Morelli, C. Amantini, S. Liberati, M. Santoni, L. Ricci-Vitiani, R. Pallini, and G. Santoni, *Cannabidiol stimulates Aml-1a-dependent glial differentiation and inhibits glioma stem-like cells proliferation by inducing autophagy in a TRPV2-dependent manner.* Int J Cancer, 2015. **137**(8): p. 1855-69

193. Armstrong, J.L., D.S. Hill, C.S. McKee, S. Hernandez-Tiedra, M. Lorente, I. Lopez-Valero, M. Eleni Anagnostou, F. Babatunde, M. Corazzari, C.P. Redfern, G. Velasco, and P.E. Lovat, *Exploiting cannabinoid-induced cytotoxic autophagy to drive melanoma cell death.* J Invest Dermatol, 2015. **135**(6): p. 1629-37.

194. Vara, D., M. Salazar, N. Olea-Herrero, M. Guzman, G. Velasco, and I. Diaz-Laviada, *Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy.* Cell Death Differ, 2011. **18**(7): p. 1099-111.

195. Vara, D., C. Morell, N. Rodriguez-Henche, and I. Diaz-Laviada, *Involvement of PPARgamma in the antitumoral action of cannabinoids on hepatocellular carcinoma.* Cell Death Dis, 2013. **4**: e618.

196. Pellerito, O., A. Notaro, S. Sabella, A. De Blasio, R. Vento, G. Calvaruso, and M. Giuliano, *WIN induces apoptotic cell death in human colon cancer cells through a block of autophagic flux dependent on PPARgamma down-regulation.* Apoptosis, 2014. **19**(6): p. 1029-42.

197. Notaro, A., S. Sabella, O. Pellerito, R. Di Fiore, A. De Blasio, R. Vento, G. Calvaruso, and M. Giuliano, *Involvement of PAR-4 in cannabinoid-dependent sensitization of osteosarcoma cells to TRAIL-induced apoptosis.* Int J Biol Sci, 2014. **10**(5): p. 466-78.

198. Torres, S., M. Lorente, F. Rodriguez-Fornes, S. Hernandez-Tiedra, M. Salazar, E. Garcia-Taboada, J. Barcia, M. Guzman, and G. Velasco, *A combined preclinical therapy of cannabinoids and temozolomide against glioma.* Mol Cancer Ther, 2011. **10**(1): p. 90-103.

199. Donadelli, M., I. Dando, T. Zaniboni, C. Costanzo, E. Dalla Pozza, M.T. Scupoli, A. Scarpa, S. Zappavigna, M. Marra, A. Abbruzzese, M. Bifulco, M. Caraglia, and M. Palmieri, *Gemcitabine/cannabinoid combination triggers autophagy in pancreatic cancer cells through a ROS-mediated mechanism.* Cell Death Dis, 2011. **2**: e152.

200. Emery, S.M., M.R. Alotaibi, Q. Tao, D.E. Selley, A.H. Lichtman, and D.A. Gewirtz, *Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells.* J Pharmacol Exp Ther, 2014. **348**(2): p. 293-302.

201. De Falco, M., R. Penta, V. Laforgia, L. Cobellis, and A. De Luca, *Apoptosis and human placenta: expression of proteins belonging to different apoptotic pathways during pregnancy.* J Exp Clin Cancer Res, 2005. **24**(1): p. 25-33.

202. Burton, G.J. and E. Jauniaux, *What is the placenta?* Am J Obstet Gynecol, 2015. **213**(4 Suppl): S6.e1-4.

203. Velicky, P., M. Knofler, and J. Pollheimer, *Function and control of human invasive trophoblast subtypes: intrinsic vs. maternal control.* Cell Adh Migr, 2015 **(ahead of print)**

204. Lager, S. and T.L. Powell, *Regulation of nutrient transport across the placenta.* J Pregnancy, 2012. **2012**: 179827.

205. Feldt-Rasmussen, U. and E.R. Mathiesen, *Endocrine disorders in pregnancy: physiological and hormonal aspects of pregnancy.* Best Pract Res Clin Endocrinol Metab, 2011. **25**(6): p. 875-84.

206. Bambang, K.N., D.G. Lambert, P.M. Lam, S. Quenby, M. Maccarrone, and J.C. Konje, *Immunity and early pregnancy events: are endocannabinoids the missing link?* J Reprod Immunol, 2012. **96**(1-2): p. 8-18.

207. Gude, N.M., C.T. Roberts, B. Kalionis, and R.G. King, *Growth and function of the normal human placenta.* Thromb Res, 2004. **114**(5-6): p. 397-407.

208. Hamilton, W.J. and J.D. Boyd, *Development of the human placenta in the first three months of gestation.* J Anat, 1960. **94**: p. 297-328.

209. Pijnenborg, R., G. Dixon, W.B. Robertson, and I. Brosens, *Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy.* Placenta, 1980. **1**(1): p. 3-19.

210. Wennekamp, S., S. Mesecke, F. Nedelec, and T. Hiiragi, *A self-organization framework for symmetry breaking in the mammalian embryo.* Nat Rev Mol Cell Biol, 2013. **14**(7): p. 452-9.

211. Murphy, V.E., R. Smith, W.B. Giles, and V.L. Clifton, *Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus.* Endocr Rev, 2006. **27**(2): p. 141-69.

212. Nadra, K., S.I. Anghel, E. Joye, N.S. Tan, S. Basu-Modak, D. Trono, W. Wahli, and B. Desvergne, *Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta.* Mol Cell Biol, 2006. **26**(8): p. 3266-81.

213. Burton, G.J., E. Jauniaux, and A.L. Watson, *Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited.* Am J Obstet Gynecol, 1999. **181**(3): p. 718-24.

214. Caniggia, I., J. Winter, S.J. Lye, and M. Post, *Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia.* Placenta, 2000. **21 Suppl A**: p. S25-30.

215. Knofler, M. and J. Pollheimer, *IFPA Award in Placentology lecture: molecular regulation of human trophoblast invasion.* Placenta, 2012. **33 Suppl**: p. S55-62.

216. Pollheimer, J., T. Loregger, S. Sonderegger, L. Saleh, S. Bauer, M. Bilban, K. Czerwenka, P. Husslein, and M. Knofler, *Activation of the canonical wingless/T-cell factor signaling pathway promotes invasive differentiation of human trophoblast.* Am J Pathol, 2006. **168**(4): p. 1134-47.

217. Pollheimer, J., P. Haslinger, V. Fock, J. Prast, L. Saleh, K. Biadasiewicz, R. Jetne-Edelmann, G. Haraldsen, S. Haider, K. Hirtenlehner-Ferber, and M. Knofler, *Endostatin suppresses IGF-II-mediated signaling and invasion of human extravillous trophoblasts.* Endocrinology, 2011. **152**(11): p. 4431-42.

218. Moffett, A. and C. Loke, *Immunology of placentation in eutherian mammals.* Nat Rev Immunol, 2006. **6**(8): p. 584-94.

219. Huppertz, B., M. Kadyrov, and J.C. Kingdom, *Apoptosis and its role in the trophoblast.* Am J Obstet Gynecol, 2006. **195**(1): p. 29-39.

220. Ji, L., J. Brkic, M. Liu, G. Fu, C. Peng, and Y.L. Wang, *Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia.* Mol Aspects Med, 2013. **34**(5): p. 981-1023.

221. Rapino, C., N. Battista, M. Bari, and M. Maccarrone, *Endocannabinoids as biomarkers of human reproduction.* Hum Reprod Update, 2014. **20**(4): p. 501-16.

222. El-Talatini, M.R., A.H. Taylor, and J.C. Konje, *Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation.* Hum Reprod, 2009. **24**(8): p. 1989-98.

223. Habayeb, O.M., A.H. Taylor, M.D. Evans, M.S. Cooke, D.J. Taylor, S.C. Bell, and J.C. Konje, *Plasma levels of the endocannabinoid anandamide in women--a potential role in pregnancy maintenance and labor?* J Clin Endocrinol Metab, 2004. **89**(11): p. 5482-7.

224. Paria, B.C. and S.K. Dey, *Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation.* Chem Phys Lipids, 2000. **108**(1-2): p. 211-20.

225. Horne, A.W., J.A. Phillips, 3rd, N. Kane, P.C. Lourenco, S.E. McDonald, A.R. Williams, C. Simon, S.K. Dey, and H.O. Critchley, *CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy.* PLoS One, 2008. **3**(12): e3969.

226. Paria, B.C., H. Song, X. Wang, P.C. Schmid, R.J. Krebsbach, H.H. Schmid, T.I. Bonner, A. Zimmer, and S.K. Dey, *Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation.* J Biol Chem, 2001. **276**(23): p. 20523-8.

227. Sun, X. and S.K. Dey, *Aspects of endocannabinoid signaling in periimplantation biology.* Mol Cell Endocrinol, 2008. **286**(1-2 Suppl 1): p. S3-11.

228. Wang, H., H. Xie, Y. Guo, H. Zhang, T. Takahashi, P.J. Kingsley, L.J. Marnett, S.K. Das, B.F. Cravatt, and S.K. Dey, *Fatty acid amide hydrolase deficiency limits early pregnancy events.* J Clin Invest, 2006. **116**(8): p. 2122-31.

229. Helliwell, R.J., L.W. Chamley, K. Blake-Palmer, M.D. Mitchell, J. Wu, C.S. Kearn, and M. Glass, *Characterization of the endocannabinoid system in early human pregnancy.* J Clin Endocrinol Metab, 2004. **89**(10): p. 5168-74.

230. Wang, H. and S.K. Dey, *Lipid signaling in embryo implantation.* Prostaglandins Other Lipid Mediat, 2005. **77**(1-4): p. 84-102.

231. Yang, Z.M., B.C. Paria, and S.K. Dey, *Activation of brain-type cannabinoid receptors interferes with preimplantation mouse embryo development.* Biol Reprod, 1996. **55**(4): p. 756-61.

232. Bari, M., N. Battista, V. Pirazzi, and M. Maccarrone, *The manifold actions of endocannabinoids on female and male reproductive events.* Front Biosci (Landmark Ed), 2011. **16**: p. 498-516.

233. Maccarrone, M., T. Bisogno, H. Valensise, N. Lazzarin, F. Fezza, C. Manna, V. Di Marzo, and A. Finazzi-Agro, *Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer.* Mol Hum Reprod, 2002. **8**(2): p. 188-95.

234. Fonseca, B.M., G. Correia-da-Silva, M. Almada, M.A. Costa, and N.A. Teixeira, *The Endocannabinoid System in the Postimplantation Period: A Role during Decidualization and Placentation.* Int J Endocrinol, 2013. **2013**: p. 510-40.

235. Fonseca, B.M., G. Correia-da-Silva, A.H. Taylor, P.M. Lam, T.H. Marczylo, J.C. Konje, and N.A. Teixeira, *Characterisation of the endocannabinoid system in rat haemochorial placenta.* Reprod Toxicol, 2012. **34**(3): p. 347-56.

236. Fonseca, B.M., G. Correia-da-Silva, A.H. Taylor, J.C. Konje, S.C. Bell, and N.A. Teixeira, *Spatio-temporal expression patterns of anandamide-binding receptors in rat implantation sites: evidence for a role of the endocannabinoid system during the period of placental development.* Reprod Biol Endocrinol, 2009. **7**:121.

237. Fonseca, B.M., G. Correia-da-Silva, A.H. Taylor, P.M. Lam, T.H. Marczylo, J.C. Konje, S.C. Bell, and N.A. Teixeira, *N-acylethanolamine levels and expression of their metabolizing enzymes during pregnancy.* Endocrinology, 2010. **151**(8): p. 3965-74.

238. Costa, M.A., E. Keating, B.M. Fonseca, N.A. Teixeira, and G. Correia-da-Silva, *2-Arachidonoylglycerol impairs human cytotrophoblast cells syncytialization: influence of endocannabinoid signalling in placental development.* Mol Cell Endocrinol, 2015. **399**: p. 386-94.

239. Mesiano, S. and T.N. Welsh, *Steroid hormone control of myometrial contractility and parturition.* Semin Cell Dev Biol, 2007. **18**(3): p. 321-31.

240. Wang, H., H. Xie, and S.K. Dey, *Loss of cannabinoid receptor CB1 induces preterm birth.* PLoS One, 2008. **3**(10): e3320.

241. Mitchell, M.D., T.A. Sato, A. Wang, J.A. Keelan, A.P. Ponnampalam, and M. Glass, *Cannabinoids stimulate prostaglandin production by human gestational tissues through a tissue- and CB1-receptor-specific mechanism.* Am J Physiol Endocrinol Metab, 2008. **294**(2): E352-6.

242. Sun, X. and S.K. Dey, *Synthetic cannabinoids and potential reproductive consequences.* Life Sci, 2014. **97**(1): p. 72-7.

243. Schneider, M., *Cannabis use in pregnancy and early life and its consequences: animal models.* Eur Arch Psychiatry Clin Neurosci, 2009. **259**(7): p. 383-93.

244. Morris, C.V., J.A. DiNieri, H. Szutorisz, and Y.L. Hurd, *Molecular mechanisms of maternal cannabis and cigarette use on human neurodevelopment.* Eur J Neurosci, 2011. **34**(10): p. 1574-83.

245. Campolongo, P., V. Trezza, P. Ratano, M. Palmery, and V. Cuomo, *Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents.* Psychopharmacology (Berl), 2011. **214**(1): p. 5-15.

246. Huizink, A.C., *Moderate use of alcohol, tobacco and cannabis during pregnancy: new approaches and update on research findings.* Reprod Toxicol, 2009. **28**(2): p. 143-51.

247. Lee, C.C. and C.N. Chiang, *Maternal-fetal transfer of abused substances: pharmacokinetic and pharmacodynamic data.* NIDA Res Monogr, 1985. **60**: p. 110-47.

248. Metz, T.D. and E.H. Stickrath, *Marijuana use in pregnancy and lactation: a review of the evidence.* Am J Obstet Gynecol, 2015. **S0002-9378**(15): p. 501-3.

249. Heuberger, J.A., Z. Guan, O.O. Oyetayo, L. Klumpers, P.D. Morrison, T.L. Beumer, J.M. van Gerven, A.F. Cohen, and J. Freijer, *Population pharmacokinetic model of THC integrates oral, intravenous, and pulmonary dosing and characterizes short- and long-term pharmacokinetics.* Clin Pharmacokinet, 2015. **54**(2): p. 209-19.

250. Westin, A.A., M.A. Huestis, K. Aarstad, and O. Spigset, *Short communication: Urinary excretion of 11-nor-9-carboxy-Delta(9)-tetrahydrocannabinol in a pregnant*

woman following heavy, chronic cannabis use. J Anal Toxicol, 2009. **33**(9): p. 610-4.

251. Costa, M.A., B.M. Fonseca, F. Marques, N.A. Teixeira, and G. Correia-da-Silva, *The psychoactive compound of Cannabis sativa, Delta(9)-tetrahydrocannabinol (THC) inhibits the human trophoblast cell turnover.* Toxicology, 2015. **334**: p. 94-103.

252. Forray, A. and D. Foster, *Substance Use in the Perinatal Period.* Curr Psychiatry Rep, 2015. **17**(11): p. 91.

253. Goldschmidt, L., N.L. Day, and G.A. Richardson, *Effects of prenatal marijuana exposure on child behavior problems at age 10.* Neurotoxicol Teratol, 2000. **22**(3): p. 325-36.

254. Huizink, A.C. and E.J. Mulder, *Maternal smoking, drinking or cannabis use during pregnancy and neurobehavioral and cognitive functioning in human offspring.* Neurosci Biobehav Rev, 2006. **30**(1): p. 24-41.

255. Huffman, J.W. and L.W. Padgett, *Recent developments in the medicinal chemistry of cannabimimetic indoles, pyrroles and indenes.* Curr Med Chem, 2005. **12**(12): p. 1395-411.

256. Mereu, G., M. Fa, L. Ferraro, R. Cagiano, T. Antonelli, M. Tattoli, V. Ghiglieri, S. Tanganelli, G.L. Gessa, and V. Cuomo, *Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4915-20.

257. Castelli, M.P., A. Paola Piras, A. D'Agostino, F. Pibiri, S. Perra, G.L. Gessa, M. Maccarrone, and M. Pistis, *Dysregulation of the endogenous cannabinoid system in adult rats prenatally treated with the cannabinoid agonist WIN 55,212-2.* Eur J Pharmacol, 2007. **573**(1-3): p. 11-9.

258. Pattillo, R.A. and G.O. Gey, *The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro.* Cancer Res, 1968. **28**(7): p. 1231-6.

259. Kliman, H.J., J.E. Nestler, E. Sermasi, J.M. Sanger, and J.F. Strauss, 3rd, *Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae.* Endocrinology, 1986. **118**(4): p. 1567-82.

260. Keating, E., P. Goncalves, C. Lemos, F. Costa, I. Campos, S.B. Smith, C.C. Bridges, and F. Martel, *Progesterone inhibits folic acid transport in human trophoblasts.* J Membr Biol, 2007. **216**(2-3): p. 143-52.

261. Huppertz, B. and J.C. Kingdom, *Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis.* J Soc Gynecol Investig, 2004. **11**(6): p. 353-62.

262. Huppertz, B., D. Ghosh, and J. Sengupta, *An integrative view on the physiology of human early placental villi.* Prog Biophys Mol Biol, 2014. **114**(1): p. 33-48.

263. Jerzak, M. and P. Bischof, *Apoptosis in the first trimester human placenta: the role in maintaining immune privilege at the maternal-foetal interface and in the trophoblast remodelling.* Eur J Obstet Gynecol Reprod Biol, 2002. **100**(2): p. 138-42.

264. Sharp, A.N., A.E. Heazell, I.P. Crocker, and G. Mor, *Placental apoptosis in health and disease.* Am J Reprod Immunol, 2010. **64**(3): p. 159-69.

265. Chen, Y. and J. Buck, *Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism.* J Pharmacol Exp Ther, 2000. **293**(3): p. 807-12.

266. Khare, M., A.H. Taylor, J.C. Konje, and S.C. Bell, *Delta9-tetrahydrocannabinol inhibits cytotrophoblast cell proliferation and modulates gene transcription.* Mol Hum Reprod, 2006. **12**(5): p. 321-33.

267. Ruiz, L., A. Miguel, and I. Diaz-Laviada, *Delta9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism.* FEBS Lett, 1999. **458**(3): p. 400-4.

268. Keating, E., P. Goncalves, I. Campos, F. Costa, and F. Martel, *Folic acid uptake by the human syncytiotrophoblast: interference by pharmacotherapy, drugs of abuse and pathological conditions.* Reprod Toxicol, 2009. **28**(4): p. 511-20.

269. Ellert-Miklaszewska, A., B. Kaminska, and L. Konarska, *Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein.* Cell Signal, 2005. **17**(1): p. 25-37.

270. Giuliano, M., O. Pellerito, P. Portanova, G. Calvaruso, A. Santulli, A. De Blasio, R. Vento, and G. Tesoriere, *Apoptosis induced in HepG2 cells by the synthetic cannabinoid WIN: involvement of the transcription factor PPARgamma.* Biochimie, 2009. **91**(4): p. 457-65.

271. Sarfaraz, S., F. Afaq, V.M. Adhami, A. Malik, and H. Mukhtar, *Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest.* J Biol Chem, 2006. **281**(51): p. 39480-91.

272. Park, J.M., X.S. Xian, M.G. Choi, H. Park, Y.K. Cho, I.S. Lee, S.W. Kim, and I.S. Chung, *Antiproliferative mechanism of a cannabinoid agonist by cell cycle arrest in human gastric cancer cells.* J Cell Biochem, 2011. **112**(4): p. 1192-205.

273. Qamri, Z., A. Preet, M.W. Nasser, C.E. Bass, G. Leone, S.H. Barsky, and R.K. Ganju, *Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer.* Mol Cancer Ther, 2009. **8**(11): p. 3117-29.

274. Wasik, A.M., S. Almestrand, X. Wang, K. Hultenby, A.L. Dackland, P. Andersson, E. Kimby, B. Christensson, and B. Sander, *WIN55,212-2 induces cytoplasmic vacuolation in apoptosis-resistant MCL cells.* Cell Death Dis, 2011. **2**: e225.

275. Moghadam, K.K., C.A. Kessler, J.K. Schroeder, A.R. Buckley, A.K. Brar, and S. Handwerger, *Cannabinoid receptor I activation markedly inhibits human decidualization.* Mol Cell Endocrinol, 2005. **229**(1-2): p. 65-74.

276. Koch, M., S. Kreutz, C. Bottger, U. Grabiec, C. Ghadban, H.W. Korf, and F. Dehghani, *The cannabinoid WIN 55,212-2-mediated protection of dentate gyrus granule cells is driven by CB1 receptors and modulated by TRPA1 and Cav 2.2 channels.* Hippocampus, 2011. **21**(5): p. 554-64.

277. Siegmund, S.V., T. Qian, S. de Minicis, J. Harvey-White, G. Kunos, K.Y. Vinod, B. Hungund, and R.F. Schwabe, *The endocannabinoid 2-arachidonoyl glycerol induces death of hepatic stellate cells via mitochondrial reactive oxygen species.* Faseb j, 2007. **21**(11): p. 2798-806.

278. Malhotra, J.D. and R.J. Kaufman, *Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?* Antioxid Redox Signal, 2007. **9**(12): p. 2277-93.

279. Nishitoh, H., *CHOP is a multifunctional transcription factor in the ER stress response.* J Biochem, 2012. **151**(3): p. 217-9.

280. Kim, A.D., S.R. Madduma Hewage, M.J. Piao, K.A. Kang, S.J. Cho, and J.W. Hyun, *Esculetin induces apoptosis in human colon cancer cells by inducing endoplasmic reticulum stress.* Cell Biochem Funct, 2015. **33**(7): p. 487-94

281. Zhang, L., X. Cheng, Y. Gao, J. Bao, H. Guan, R. Lu, H. Yu, Q. Xu, and Y. Sun, *Induction of ROS-independent DNA damage by curcumin leads to G2/M cell cycle arrest and apoptosis in human papillary thyroid carcinoma BCPAP cells.* Food Funct, 2015. **(ahead of print)**.

Appendix